ACTA
PATHOLOGICA
ET MICROBIOLOGICA
SCANDINAVICA
VOL 80A

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

Section A PATHOLOGY

VOL. 80 A, FASC. 1-6, 1972

MUNKSGAARD COPENHAGEN

Munksgaard
International Booksellers and Publishers, Ltd
47, Prags Boulevard, DK-2300 Copenhagen S, Denmark



PRINTED IN DENMARK
VALD PEDERSENS BOGTRYKKERI A/S
COPENHAGEN

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VOL 80 A FASC 1–6 1972

SUPPLEMENTS

Acta path microbiol scand Section A 80, 1–4, 1972

SIMULTANEOUS EPIDERMOID CARCINOMA IN SITU OF THE PORTIO/ CERVIX AND THE ENDOMETRIUM OF THE UTERUS

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Two cases of epidermoid carcinoma *in situ* of the portio/cervix and the endometrium of the uterine body of a 58 year-old and a 76 year old woman are presented The histology is described and the differentiation of the precancerous epithelium is discussed on the basis of the literature The indifferent basal-cell is suggested to be the cell of origin of physiological as well as pathological proliferations which to some extend may be influenced by the stromal changes

Pure squamous cell carcinoma arising from the endometrium of the uterine body is a rare lesion as pointed out by *Willis* 1967, and *Hartig & Gore* 1960 This is in contrast to the squamous metaplastic elements often found in endometrial adenocarcinomas

Only a small number of primary epidermoid carcinomas have been seen by *Novak & Woodruff* 1967, in older women with preceding metaplasia of the columnar epithelium into squamous cell epithelium As the number of reports on cases of epidermoid carcinoma in this location is limited the present cases are reported

CASE REPORTS

1) A 76 year-old woman who was admitted to hospital on account of postmenopausal metorrhagia described as a brownish vaginal discharge Menopause 30 years ago one natural birth The patient has never been hospitalized or suffered from any essential disease Examination of routine smears showed tumour cells and biopsy gave evidence of carcinoma *in situ* The patient was treated by total hysterectomy Metastases were not observed

Gross Pathology The uterus measured 5×3×3 cm The uterine cavity was 3,5 cm long and the portio measured 2 cm in diameter There was no macroscopic signs of tumour tissue

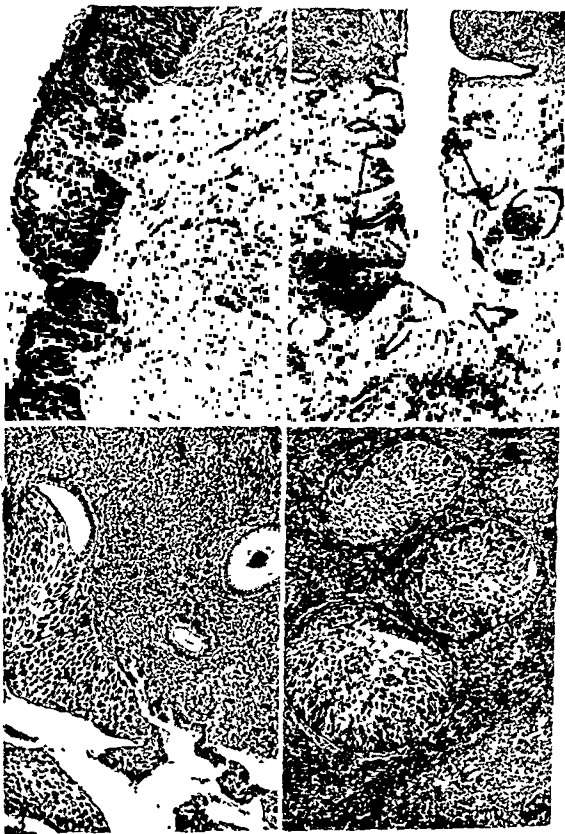
2) A 58 year old woman hospitalized on account of postmenopausal vaginal discharge of 6 months duration Dilatation of the cervival canal disclosed moderate pyometra Portio biopsy showed carcinoma in situ The patient was treated by conization followed by total hysterectomy and metastases were not found

Gross Pathology The uterus measured 7×5×2 cm There was no evidence of tumour tissue The uterine cavity was 4 5 cm long and the portio measured about 2 cm in diameter

METHODS

Both specimens were after formalin fixation, cut into several sections comprising portio, cervix and the endometrium The tissue was, after paraffin embedding and cutting stained by haematoxylin/eosin

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HISTOLOGY

Fig 1 demonstrates a carcinoma *in situ* in the surface epithelium of the portio, characterized by increased cellularity, disorderly arrangement of cells loss of stratification, marked hyperchromatic nuclei and a number of atypical mitoses Disaray of cells and individual cell keratinization were noted but no signs of stromal invasion were present

The cervical mucosa was partly covered by epithelium which was both of columnar and squamous type The number of glands was reduced and the surrounding stroma appeared fibrotic A few cervical glands were dilatated and contained mucus others were filled with dysplastic squamous epithelium Areas in which the columnar epithelium was covered by squamous epithelium were not in evidence, and there was no signs of stromal invasion

The endometrium of the uterine body of both cases appeared as illustrated in Fig 2 The surface was lined with both low columnar and squamous epithelium the latter showing light to severe dysplastic changes with an increased number of mitoses and loss of normal maturity The areas covered by cylindric epithelium were scarce and in no case covered by squamous epithelium The number of endometrial glands was reduced although still within the normal range of the postmenopausal age, but in several glands the columnar epithelium was replaced by a metaplastic squamous epithelium showing varying degrees of dysplasia including carcinoma *in situ* as demonstrated in Figs 3 and 4 The endometrial stroma was characterized by fibrosis and around the squamous dysplastic epithelium, infiltration with lymphocytes, plasmacells and histiocytic cells was present

DISCUSSION

The literature on the occurrene of squamous epithelium in the endometrium of the uterine body has been thoroughly reviewed by *Baggish & Woodruff* 1967 They consider the presence of squamous metaplasia of the endometrium a common finding In 100 randomly selected cases they found foci of metaplasia to be present in 95 of the endometrial biopsies, but no comments on the possible admixture with squamous epithelium from the cervix/portio area were given

Baggish & Woodruff, l.c, quote from the literature 13 documented cases of primary squamous carcinoma in the uterine endometrium and support the correctness of the criteria originally set up for the lesion by *Fluhmann* 1928

1) No co existing adenocarcinoma,
2) No demonstrable connection between the tumour and the stratified epithelium of the cervix,
3) The cervix must be examined completely to eliminate the existence of a primary site in the organ

Lahm 1928, has surveyed the German literature and divided the cases more broadly into 3 categories

1) Arising primarily in the cervix and spreading upwards into the endometrium,
2) Probably arising primarily in the uterine body spreading downwards to involve the cervix,
3) Undetermined primary site,

the last classification thus being less clear cut than that suggested by *Fluhmann*

In the opinion of the author, the two present cases cannot be classified as a primary

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Fig 1 shows carcinoma in situ in the surface epithelium of the portio (H & E × 100)

Fig 2 demonstrates a section from the fundus of the uterine body The glands are lined partly with squamous partly with columnar epithelium Some glands (arrows) are filled with dysplastic squamous epithelium (H & E × 45)

Figs 3 and 4 [illegible] is lined [illegible] ly with c[illegible] tic stroma [illegible] by lymphocytes and plasma cells (H & E × 100)

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A CASE OF CROHN'S DISEASE OF THE COLON ASSOCIATED WITH ADENOCARCINOMA EXTENDING FROM CARDIA TO THE ANUS

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A case of Crohn's disease of the colon coexisting with an alimentary, differentiated adeno carcinoma extending from cardia to the anus is reported The criterions for the diagnosis morbus Crohn and the association between the two diseases are discussed

Adenocarcinoma of the colon and the small intestine associated with Crohn's disease has in recent years been reported with increasing frequency, but no firm association between the two diseases has been established as between ulcerative proctocolitis and carcinoma About twenty cases of carcinoma of the small intestine (*Wyatt* 1969, *Farmer et al* 1970) and about ten cases of colon carcinomas (*Jones* 1969) coinciding with Crohn's disease have been reported Furthermore reticulosarcoma (*Huges* 1955, *Wyburn-Mason* 1968) and carcinoid tumours (*Wood et al* 1970) coexisting with morbus Crohn have been described

Previously reported carcinomas have mostly been solitary tumours Multifocal carcinomas coinciding with Crohn's disease have been reported in a few cases (*Bersack et al* 1958) but to our knowledge, cases of widespread diffuse carcinoma of the intestinal canal associated with Crohn's disease have not been described

Received 26 x 71

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CASE REPORT

A 70 year old man, born 1897 In May 1965 a diagnosis of tardive cutaneous porphyria was made The patient was treated with prednisone Due to positive benzidine reactions an X ray examination of the colon was performed disclosing slight diverticulosis Proctoscopy revealed a few small apparentley benign, polyps (no biopsy) but no signs of malignancy or stenosis

In the following two years he had irregular defecation He was seen at this hospital in February 1967, feeling tired but otherwise in a good state Haemoglobin amounted to 12,4 g/100 ml and erythrocyte sedimentation rate to 60 mm/1 hour Proctoscopy revealed several, apparently benign, polyps and 15 cm from the anus a papillomatous area with stenosis Biopsy (25/2 1967) disclosed Crohn's disease and X ray examination showed segmentally localized stenoses of the Crohn type at the recto sigmoidal junction, near the left flexure and in the transversal colon He was treated with cortisone locally and with salazopyrine and the stenosis dimished

The patient was followed proctoscopically and in the beginning of 1968 the rectal stenosis progressed and a large ulcer appeared A biopsy (8/3-1968) still showed Crohn's disease without

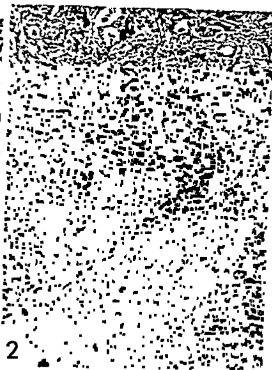





Fig 1 Section representative of both rectal biopsies Several granulomas but no carcinomatous infiltrations are seen *(H & E × 40)*

Fig 2 Section from the caecal biopsy Sarcoid granulomas and adenocarcinomatous tissue are demonstrated *(H & E × 100)*

Fig 3 Both granulomas and carcinoma in a post mortem section from the sigmoideum (H & E × 25)

Fig 4 Section from the pyloric muscle demonstrating differentiated adenocarcinoma *(H & E × 40)*

malignancy A few months later he suddenly developed abdominal pain and was admitted to the hospital where a diagnosis of ileus of the small intestine was made He was treated conservatively but since subileus persisted and he had lost 12 5 kg in weight an exploratory laparotomy was performed Extensive infiltrations were found in the upper part of the rectum in the caecum and in the cardiac part of the stomach Besides, several stenoses a few centimeters long were seen in the small intestine and in the transversal colon There was infiltration in the omentum majus and ascites Biopsies (7/1–1969) from the omentum and the wall of the caecum disclosed adenocarcinoma, the latter combined with signs of Crohn s disease Radical surgery proved impossible but three palliative shunt operations bypassing stenotic parts were performed He was discharged and died in his home one and a half month later No full necropsy could be performed but since the patient, by will had left his body to the Institute of Normal Anatomy we were able to take biopsies from all parts of the alimentary tract.

PATHOLOGICAL EXAMINATION

The two rectal biopsies (27/2–1967 & 11/3 1968) (Fig 1) were alike The mucosa was well preserved in both except for small ulcerations, heavily infiltrated with lymphocytes and plasma cells The submucosa and the muscularis showed a large number of lymphocytic foci and several granulomas all of the sarcoid type Special staining showed no tubercle bacilli Serial sections of both biopsies did not reveal malignant changes

The biopsy (7/1–1969) from the caecum (Fig 2) was heavily infiltrated with carcinoma In some areas well differentiated glands were seen in others the tumour cells were arranged in solid cords Two large collections of typical granulomas were seen in the submucosa

The biopsy from the omentum revealed a well differentiated adenocarcinoma but no signs of Crohn s disease

As mentioned biopsies were taken post mortem from all parts of the gastro intestinal tract In all sections from the rectum and colon (Fig 3), from the small intestine and from the stomach (Fig 4), carcinomatous changes were seen The tumour tissue consisted of thin walled gland imitations but also more solid clusters of cells were seen No signet ring cells were disclosed Small amounts of connective tissue surrounded the malignant glands, but the tumour tissue could not be characterized as sclerotic nor had it the appearance of a typical carcinoma of the colon It was impossible to decide the starting point of the tumour Sarcoid granulomas, typically transmurally arranged, were seen in all sections from the colon (Fig 3) and rectum but not in more proximal locations

With a view to metastases a few lymph nodes from the mesentery were sectioned but neither tumour tissue nor granulomas were found

The liver surface was normal The thoracic cavity was not opened

COMMENT

The diagnosis morbus Crohn was based mainly on the microscopic findings from the rectal biopsies which were close to pathognomonic and correspond with both clinical and radiological findings It is well known that sarcoid lesions may occasionally be found in the immediate neighbourhood of malignant neoplasms (*Gresham & Ackerley* 1958, *Gregorrie et al* 1962), but in our case serial sections disclosed no signs of tumour tissue and malignancy was not disclosed until two years later

The carcinoma occurred in all sections from cardia to the anus It was remarkably diffuse but even in the stenosed areas only a moderate formation of connective tissue had taken place A few cases of carcinomas diffusely involving the alimentary tract have previously been described (*Fernat et al* 1965 *Correia et al* 1968) but they have all been of the linitis plastica type

The relation between Crohn s disease and carcinoma is obscure As mentioned by *Jones* (1969), an occurrence of the two diseases in one and the same patient may be caused by an individual predisposition to both He reported a case of rectal carcinoma later developing into ileitis terminalis Similar cases were described by *Cornes & Stecher* (1961)

and *Lennard-Jones* & *Stalder* (1967) and cases of Crohn's disease in which carcinomas developed in different parts of the colon have been described by *Davis* & *Caley* (1960) and *Hank* & *Turnbull* (1966)

It has never been convincingly demonstrated that morbus Crohn will cause carcinoma, unlike ulcerative colitis, where the risk of malignant transformation is an established fact (*Eduard* & *Truelove* 1964) and in which precancerous changes in the epithelium had been demonstrated (*Morson* & *Pang* 1967) A single case of ulcerative colitis complicated with diffuse colonic carcinosis has been reported from this country (*Bryld 1970*)

As pointed out by *Correia et al* (1968) and *Maratka et al* (*1970*) the differential diagnosis between morbus Crohn and a widespread diffuse carcinoma with stenosis may be very difficult or impossible Only sufficient biopsies will secure a correct interpretation

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Acta path microbiol scand Section A 80, 9 16, 1972

THE ISO—AND ALLOTRANSPLANTED RAT HEART

Histological, Electrocardiographic and Serological Observations

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The morphological changes in 18 isografted and 108 allografted hearts have been studied in three inbred strains of rats Focal cellular infiltrates were occasionally present in isografts in or near areas of infarction, and showed regressive tendency during the weeks after transplantation Lymphoid cell infiltration in allografts was observed from the third day and increased progressively till a few days before terminal rejection, at which time extensive myocardial degeneration and oedema were the most prominent changes Electrocardiograms from isografts remained almost unaltered for prolonged periods while allografts gradually lost voltage of the R wave during rejection Haemagglutinating red cell antibodies and lymphocytotoxic antibodies were regularly produced during rejection in the combinations investigated

A surgical technique for cervical heart transplantation in rats was described recently (4) The present report describes the histological, electrocardiographic and serological changes which have been observed in heart isografted and allografted rats operated by this method

The data to be presented serve as morphological and serological standards for heart transplantations in unmodified rat recipients of three different strains studied in this laboratory

MATERIAL AND METHODS

Inbred rats of the strains Fischer (F), Wistar (Wi) and Brown Norway (BN) from 3–6 months of age and weighing 150–400 gm were used as recipients

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Donors were F, Wi and BN rats and various hybrids raised from them Donors weighing approximately 150 gm were preferred, but animals weighing up to 300 gm have served as heart donors The surgical details were exactly as described previously (4)

Transplanted hearts were removed at varying intervals after surgery (6 and 12 hours 1, 2 3, 4, 5, 6 days) and the majority of allotransplants were studied at the time of 'spontaneous rejection, as determined by total loss of electrical activity A total of 108 allotransplants and 18 isografts have been available for the histological study

Tissue for paraffin embedding was fixed in 80 per cent ethanol and routinely stained with haematoxylin-eosin and methyl green and pyronine

Electrocardiograms were taken on ether anaesthetized rats

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Bloodsamples were obtained from tail veins of rats or drawn by aortic puncture The blood was allowed to clot at room temperature for 30 min

ntes and serum collected following centrifugations and stored at —25° C until used

Haemagglutination test Erythrocytes from the donor animals were collected in sodium citrate washed three times in physiological saline and suspended in 20 per cent bovine albumin to give a 5 per cent suspension

Serial dilution of heat inactivated serum in 2 per cent dextran in saline was made One drop of the erythrocyte suspension was incubated in tubes with two drops of diluted serum for 2 hours at 37° C The tubes were read for agglutination by inspection of the erythrocyte pellet during gentle shaking and scored as + to + + + +

Lymphocytotoxic test Tests were performed using microtechniques Pure lymphocyte suspensions were prepared from defibrinated blood obtained by aortic puncture by use of a modification of the method described by Boyum (2) Lymphocytes were washed two times in Hanks balanced salt solution and suspended in equal volumes of fresh normal rat serum and guinea pig serum at a concentration of approximately 2 000 cells per µl One µl of the cellsuspension was mixed under oil with one µl serum to be tested Following incubation for 2 hours at 37° C tryphan blue was added and the percentage kill of cells was determined after 30 minutes under inverted microscope

Complement Fixation Test

Microtechnique was used as a modification of that described in an earlier study (12) Thrombocytes were prepared from citrate blood and washed in barbital buffer and suspended in an 0 1 per cent Na azide barbital buffer solution at a concentration of approximately 1 million per µl suspensions were stored at 4° C for seven days before use Serum was heat inactivated and diluted in barbital buffer the lowest dilution being 1 5

Diluted guinea pig serum was used as source of complement Two haemolytic units were used for each test

Sensitized sheep red blood cells at a concentration of 200 000 pr µl served as indicator for complement

Platelets (2 µl), diluted serum (2 µl) and complement (2 µl) were mixed under oil in Falcon plastic trays and incubated for one hour at 37° C 2 µl of sensitized sheep red blood cells were added to each well and plates were incubated for 30 minutes at 37° C The degree of haemolysis was determined under microscope Appropriate anticomplementary controls and negative serum and platelet controls were run parallel to each serum to be tested for complement fixing platelet antibodies

RESULTS

The survival times of allotransplanted hearts in the different recipient donor combinations are summerized in Table 1

Isotransplanted hearts have survived in Wi F and BN rats for 290, 422 and 210 days and are still beating in good condition

Histology Allografted hearts in the different rat strain combinations showed the same morphological alterations in principle but a tendency towards less vigorous reactions in Wistar hearts transplanted to Fischer recipients was noted in comparison with hearts in the other combinations investigated

Allo and isotransplanted hearts removed

TABLE 1 *Survival Times of Allografted Hearts in Unmodified Rat Recipients*

Recipient strain	Donor strain	Survival range days	Number of transplants	Mean survival days ± SEM	SD
Wi	F and (Wi × F)F_1	7–16	42	9 4 ± 0 27	1 76
Wi	(Wi × BN)F_1	6 13	20	8 7 ± 0 39	1 75
F	Wi	7 11	17	8 6 ± 0 29	1 18
BN	(F × BN)F_1	8 9	2	8 5	
BN	Wi	7–11	2	9 0	

Wi Wistar
F Fischer
BN Brown Norway
F_1 First generation cross
SEM Standard error of the mean
SD Standard deviation

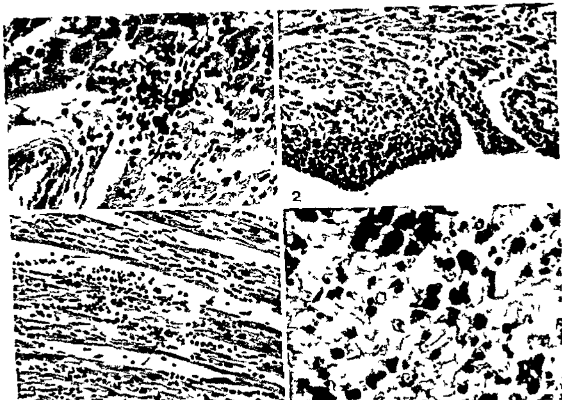


Fig 1 Perivascular cell infiltrate in an allotransplant after 3 5 days (haematoxylin and eosin × 400)
Fig 2 Heavy lymphocyte infiltrates are present in the endocardium of the right ventricle in an allo graft 4 5 days after transplantat on (haematoxylin and eosin × 250)
Fig 3 Allotransplant 5 days post transplantation Linear interstitial lymphoid cell infiltrates are present (haematoxyl n and eosin × 250)
Fig 4 Severe degeneration of myocard um (myocytolysis) with few infiltrating cells at terminal rejec tion in allograft day 8 (haematoxylin and eosin × 400)

within 12 hours after transplantation appear ed entirely normal as compared to rat hearts taken from ether killed normal rats Grafts removed later than 12 hours all showed signs of mechanical injury consisting in epicardial fibrinous exudation and subepicardial cell aggregates At 3 days after surgery accumu lation of lymphoid cells appeared at first in the allotransplant Characteristically, these cells were distributed in small clusters around small and medium sized vessels (Fig 1) and could also be seen adjacent to the endocar dium of the right ventricle (Fig 2) A few blast cells with pyroninophilic cytoplasma was often present in this early infiltrate

Allohearts removed four days after trans plantation revealed a slight progression of the lymphoid infiltrates mentioned and the first interstitially localized lymphocytes could be seen between myofibres (Fig 3) Beyond 4 days until two days before terminal rejec tion the spread of infiltrating mononuclear cells in allografts was progressive and ac companied by degenerative parenchymal changes

Focal areas in the myocardium almost free from lymphocytes but with loss of myo fibres and proliferation of macrophages and fibroblasts were not rarely found from day 3 and on (as in isografts)

Degenerative changes in transplants at terminal rejection were infarctions with cell infiltrates often including polymorphonu clear leucocytes and loss and atrophi of



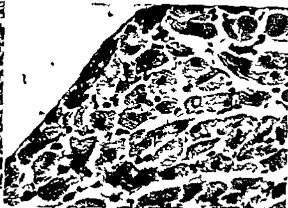


Fig 5 Isotransplant after 5 days showing cellular infiltrates present in an infarcted myocardial area (haematoxylin and eosin × 250)
Fig 6 Endo and myocardium of the right ventricle in an isotransplant at 5 days following transplantation (haematoxylin and eosin × 400)

myocardial cells (myocytolysis) with or without surrounding mononuclear cells (Fig 4) Interstitial oedema and occasionally foci with haemorrhages accompanied the destruction of myofibres at rejection The lymphoid cell infiltrates tended to decrease in density at the endpoint of rejection as compared with the precedent days There were several instances in which focal areas in the graft contained normal looking myofibres surrounded by infiltrating cells despite total loss of electrical activity Only very few mature pyroplasmacells but a moderate number young plasmacells were seen at the time of rejection

Isotransplants were morphologically identical to the allografts in the period of the first 2 days From day 3 to 5 and on focal infarctions with infiltrating connective tissue cells (but not the characteristic lymphocyte infiltrate) was not uncommon (Fig 5) while quite normal myocardial areas were found in between (Fig 6) Smaller infarctions of this kind in isografts showed organisation and signs of healing when hearts were investigated at later periods and progressive destructive changes did not show up Occasionally transplants removed after 7–16 days and later were found to be totally free from histological alterations except for the subepicardial reaction

Electrocardiograms taken immediately after transplantation *often showed different ab*normalities including bradycardia conduction abnormalities and arrhythmias These changes only persisted during the first few hours, and regular rates and normal ventricular complexes were normally found during the following days

ECGs from allografts from around the fifth day and till terminal rejection showed gradually decrease in the voltage of the ventricular complexes (Fig 7) This decline in voltage developed with increasing rapidity during the terminal days and hours before total loss of electrical activity During the last periods widened and abnormal ventricular *complexes and irregular rates* were common

Elevation of ST segments were occasionally observed but upon movement of the V lead to another precordial area isoelectrical STs could be recorded

ECGs taken over isografted hearts have remained unaltered with normal complexes as late as 400 days after transplantation QRS voltage have been almost constant in these long term surviving transplants (Fig 8)

Serological Investigation

Rats carrying isografts did not develop allo antibodies and none of the rats investi



Fig 7 Electrocardiograms of an allografted heart on 1st, 6th, 7th, 8th and 9th day after transplantation Gradual decrease in R wave voltage of the transplant (T) is evident compared with the interference from the recipients heart (R) Rejection is completed at day 9, when electrical activity is totally lost

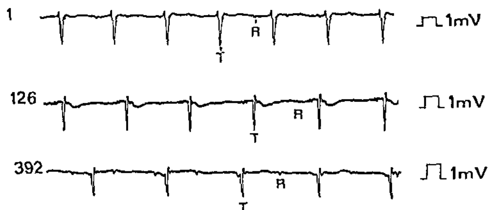


Fig 8 Electrocardiograms of an isografted heart taken 1, 126 and 392 days after transplantation R wave voltage of transplant (T) is almost constant during long periods Recipient s own heart (R) is also recorded

gated had antibodies prior to the experiments

Allotransplanted rats in the combinations examined as a general rule formed haemagglutinating and cytotoxic antibodies and these two kinds of antibodies mostly occured at the same time and roughly corresponded in potency at any given time, although exceptions to this were seen in a few instances Typical examples are shown in Fig 9 The

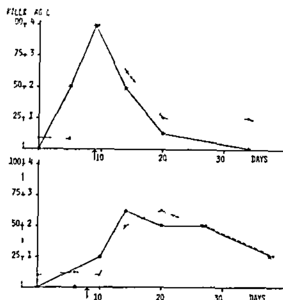


Fig 9 *Haemagglutinating red cell antibodies* (*———* and lymphocytotoxic antibodies (● - - - ●) in two heart allografted rat recipients who rejected their transplants (indicated by ↑) after 9 and 8 days

most hightitred antibodies were found in Wistar recipients receiving (WiXBN) F_1 grafts, the highest titer being 1 64 in haemagglutination and 1 32 in cytotoxicity

Complement fixing platelet antibodies could only be detected in few recipients, and did not exceed a titre of 1 10 In sera con these antibodies, strong lymphocyto ic antibodies were always present at the ame time

DISCUSSION

The earliest histological signs specific for the allografts appeared on the third day after surgery and consisted in lymphocyte infiltration primarily localized perivascularly and subendocardially in the right ventricle This observation is in good accordance with earlier studies by *Abbott et al* (1) who studied Lewis rat recipients receiving abdominal heart transplants from Lewis or Wistar Furth donors

Compared with rabbit cardiac allografts (6), rat transplants show the characteristic cell infiltrate later and the typical lymphoid cell components did never appear to be so densely represented in the infiltrates at final rejection It was striking to observe the relatively heavy cell infiltrates that were occasionally present focally in some isotransplanted hearts which also indicates possibility for a great deal of immunologically unspecific reactions in allografts The factors responsible for this are most probably mechanical injuries and focal infarctions due to anoxia and eventually air emboli and vascular spasms arising during the operative procedure

The relative decrease in density of the mononuclear cell infiltrates observed during the last days, in the period where the extensive degenerative myocardial changes occur, suggests some kind of mutual toxic substances released in the graft during rejection This is parallel to the observations done by *Shehadeh et al* (16) in rat kidney allografts and in accordance with the suggestions offered by *Abbott et al* (1)

The electrocardiographic changes recorded during the course of an allografted heart were characteristically different from those of an isograft, and ECG s have been used in later studies as a tool for the exact determination of final endpoint of rejection The decrease in R wave voltage which was concluded to be the specific parameter for the progression of rejection reaction in the hearts have been found a reliable indicator in studies on dogs (11) and rabbits (6) ST elevation as emphasized by *Lee et al* (10) to be the best indicator was found in the actual study to be an inconstant change most probably related to focal infarctions developing during rejection *Van Bekkum et al* (18) made abdominal heart transplants by use of the method of *Ono et al* (13) and found that the R wave recorded from isotransplanted heart showed gradually and considerable decrease during a period of 300 days

This was not characteristic in the present series of investigations The reason must be found in the local graft bed such as gradual increase of fibrosis surrounding an intraperitoneally situated transplant while the sub

cutaneously localized transplant permits a more direct electrocardiographic registration

The presence of humoral cytotoxic antibodies and of haemagglutinins during and following skin allograft rejection in rats has been demonstrated (8, 9, 14), and others have detected antibodies in rats carrying renal allografts appearing 3–7 days after transplantation (3, 17)

The production of these antibodies also after heart allografts in rats parallels the above observations The haemagglutinins which develop following rejection of transplants grafted across the strong histocompatibility locus in rats (Ag B locus) have been shown to be directed against red cell antigens which segregate with histocompatibility antigens This has been taken as an indication of genetic linkage or possible identity between rat red cell antigens and strong histocompatibility antigens (7, 15)

The significance of cytotoxic antibody production for the rejection reaction has been discussed elsewhere (5, 19) It was interesting to observe rats being relatively good producers of allo antibodies following challenge with a heart transplant compared with rabbits in the same situation, because rabbits in general are known to be better antibody producers than rats

CONCLUSION

The allografts in different rat strain combinations showed in principle the same typical and classical patterns of rejection dominated by infiltrating mononuclear cells in contrast to isografts Haemagglutinins and lymphocytotoxins were formed in most allografted recipients while complement fixing platelet antibodies only were seen in the sera with strongest cytotoxic antibodies The reliable ECG parameter of rejection in progress was a decline in R wave voltage

This work was supported by grants from *Kong Christian den Tiendes Fond, Danish Medical Research Council* and a scholarship under the *University of Aarhus*

I want to thank miss *A Bang* for excellent technical assistance

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cta path microbiol scand Section A 80, 17–20 1972

ON MILD DEGREES OF FLUOROSIS

2 *An Experimental, 2-Year-Study of Rats Drinking Distilled Water Containing 0, 1 or 5 ppm F*

B Sundstrom

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24 recently weaned Sprague Dawley rats were divided equally into 3 groups and given distilled drinking water *ad libitum* containing 0, 1 or 5 ppm F (as NaF), respectively Among the 13 animals that received the fluoridated water and survived the 2 year experimental period 4 developed macroradiographically demonstrable resorption cavities in their femurs Three of these animals belonged to the 1 ppm group No resorption cavities were seen in the 6 surviving, control rats There was no sign of tooth changes especially no striated depigmentation of the enamel of the incisors

Only 2 laboratory studies on fluorosis seem presently at hand, where the experimental parameters have been chosen to encompass a *long term* administration of *low levels* of fluoride Rats were used in both studies *Ramseyer et al* (1957) supplied distilled drinking water containing 0, 1, 5, or 10 ppm F *ad libitum* to groups of albino rats for – at the most – 520 days and reported loss of teeth and periodontal disease to be more frequent in the rats which received the fluoride supplementation As pertaining to the present study ,*Ramseyer et al* found no effect of fluoride at low levels on the density or the volume of the femurs of their animals They did not however, investigate the bone tissue histologically This was done microradiographically on ground sections from several bones by *Röckert* (1963) who studied Sprague Dawley rats given distilled water *ad libitum* containing 0, 1, 5, 10, 20, or 40 ppm F

Röckert killed his animals after different time intervals, the longest experimental period being 3 years, and found – in some rats – characteristic bone changes that differed among the groups of animals Those affected on higher levels of fluoride (40 and 20 ppm F) showed osteosclerosis upon 9–12 months' experimentation, but later developed resorption cavities At lower fluoride levels (5 and 1 ppm F) no osteosclerosis, but sometimes directly appearing resorption cavities occurred No such cavities were seen in the control rats

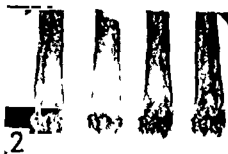
The present study was designed primarily to check again the effect of low levels of fluoride on the histology of rat femurs The results, following a 2 year experimental period with 19 rats, confirm the report by *Rockert* (1963)

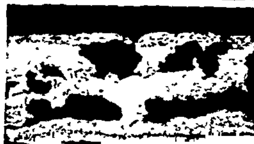
MATERIAL AND METHODS

24 recently weaned Sprague Dawley rats were divided equally into 3 groups and given distilled drinking water *ad libitum* containing 0, 1 or 5 ppm F^- (as NaF) respectively All rats were fed a diet of ordinary pellets (Ferrosan, Malmo) Following a 2 year experimental period, the distal parts of the femurs, the middle part of the verte

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Figs 1–2 Macroradiographs of 7 femurs, one from each of 7 rats that received distilled artificially fluoridated – 1 ppm – drinking water *ad libitum* for 2 years Note resorption cavities in 3 bones (from left to right, number 3, 4 and 5) × 1 5 (approx)

Fig 3 Microradiogram from one bone (no 3) in Fig 1 The resorption cavities are lined by smooth as well as scalloped contours Note periosteal and endosteal layers of lamellar bone, displaying a relative *radiolucency and larger osteocyte lacunae* × *20*

Fig 4 H and E stained section from the decalcified half of the femur in Fig 3 The resorption cavity is filled with loose connective tissue × *120*

Fig 5 Macroradiograph of a femur (bone to the left) with resorption cavities from one animal that – under similar conditions (see legend to Figs 1–2) – drank water with an F concentration of 5 ppm The right femur stems from a control rat × 1 5 (approx)

Fig 6 Ground section of an upper incisor from the 5 ppm animal with femoral resorption cavities shown above (Fig 5) Note the homogeneity of the outer pigment layer in the enamel × 50

brate columns and the upper and lower jaws were [illegible] 10 … male) and [illegible] ber of normal ones were then split longitudinally and each half again similarly photographed Ground sections, prepared from one part *of the split femurs* and from the jaws (including the teeth) were then microradiographed (Cu anode, Ni filter 12 kV/35 mA, Kodak Maximum Resolution Plates) The other part of the split femurs was decalcified in 4 N formic acid and embedded in paraffin, subsequent microtome sections being stained with haematoxylin/eosin according to v Gieson or with a *combined sequence of alcian blue* (in acetic acid) and PAS

RESULTS

5 animals did not survive for 2 years and were not included in the final analysis 2 of them were killed because of developing adenomas affecting 1 animal in the 0 and one in the 1 ppm group The cause of death in the other 3 animals – 1 in the 0 ppm group, 2 in the 5 ppm group – was not further investigated There was, however, no gross signs of either gastro-intestinal dis orders or tumour development The other 19 animals thrived well and had – in accordance with earlier investigations – normal body weights

Among the examined bones, the femurs were found to be most susceptible to pathological involvement In fact, there was only one instance, in the 5 ppm group, where the jaw bones – and then particularly the mandible – were involved None of the vertebrate columns of the experimental ani mals could with certainty, be macroradio graphically distinguished from those of the control animals As the alterations of the jaw bones were similar to those in the femurs, the more detailed analysis was restricted to the latter

Macroradiographs of 7 femurs, one from each of the surviving rats in the 1 ppm group, are shown in Figs 1 and 2 Four of these bones could not be visually or radio graphically distinguished from those of the control rats, which were all of normal ap pearance with all techniques However, 3 animals in the 1 ppm group (Figs 1 and 2) showed the presence of resorption cavities in their femoral cortices There were no ex ostoses and no gross changes in either bone or cortex widths Microradiographically the resorption cavities were outlined by both smooth and scalloped contours (Fig 3) The same technique further disclosed periosteal and also endosteal layers of lamellar bone, which varied in width among the affected animals and were characterized by a decreased mineral content and comparatively large osteocyte lacunae (Fig 3) In decalcified specimens the resorption cavities were filled with a loose connective tissue that often showed wide capillaries (Fig 4) The border of the cavities disclosed both formative (osteoid seams, osteoblasts) and resorptive (scalloped contours, multinucleated osteo clasts) features

In the 5 ppm group, 5 out of the 6 animals showed normal femurs The 6th animal had developed resorption cavities similar to those found among the animals in the 1 ppm group One of the femurs of this animal is compared with a femur of a control rat and shown in Fig 5

The applied staining techniques did not display any details in the matrix of the affected bones that distinguished them from normal bones

The teeth of all animals were normal with all techniques Especially, there was no macro scopical, striated depigmentation of the incisors of the experimental animals The pigment layer of the enamel was also found to be of homogeneous thickness in ground sections (Fig 6)

DISCUSSION

The main result of this study which comprised a small number of rats, is the appearence of resorption cavities in the femoral cortices of some experimental animals This was seen especially among those animals that received the lower fluoride concentration (1 ppm F) Similar results were already re ported in a comparable long term experi mental study by *Röckert* (1963) He found both resorption cavities and also fewer cavities at increasing (*i e* 1, 5 and 10) water fluoride levels *Röckert's* larger study, which further entailed calculations of the total fluoride intake – from drink and food – of the rats, remains largely unquoted (cf Fluorides and Human Health 1970, Fluoride in Medicine 1970)

The skeletal changes were presently re corded without concomitant, either macro or microscopical changes in the appearence of the teeth It should be emphasized that this situation contrasts sharply with the reported

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effects of fluorides in man, where several extensive studies have shown that the most susceptible system following the administration of F^- in drinking waters, is the developing tooth enamel (cf Fluorides and Human Health 1970) Hence, until, if at all, specific, technical parameters in the laboratory studies can be shown to preferably enforce skeletal changes, one has to conclude that fluoridation experiments with rats have proven to be of a limited applicability in human contexts This judgment would of course also pertain to delicate effects of fluoride on tooth development, which in rats apparently may be expressed upon an altered metabolic background

One technical parameter that should be further investigated is the choice of water In the present study and in that by *Rockert* (1963), distilled water was used as the basic vehicle for the fluoride administration *Gedalia et al* (1960) investigated the effect of water fluoridation on thyroid function, bones and teeth of rats on a low iodine diet In their study, 18 male albino rats divided into 3 groups, received drinking water containing 0 55, 1, and 10 ppm F *ad libitum* for 9 months They investigated the femurs histologically and found no structural altera† However, they used 'normal Jerusalem tap water of 0 55 ppm fluorine as the basic vehicle and 'fortified' this up to 1 and 10 ppm F, respectively for the experimental groups A special long term study, in which the effects of distilled and artificially fluoridated waters are compared with those of naturally fluoride containing waters, therefore ought to be undertaken before further pertinent analyses – of *e g* serum calcium and phosphate levels and parathyroid function – are carried through using the present experimental parameters

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RELATION BETWEEN CEASED URINE EXCRETION AND POSTNEPHRECTOMY INCREASE IN RENIN SUBSTRATE

Effects of Peritoneal Dialysis on Nephrectomized Rats and of Vesico-venous Anastomosis or Intravenous Infusion of Urine on Normal Rats

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Vesico-venous anastomosis or urine infusion in rats resulted in a marked increase in renin substrate, which increase, however, on an average was less pronounced than that found in nephrectomized rats These results do not allow an unambiguous answer to the question of the role of ceased urine excretion in the postnephrectomy increase in renin substrate in rats Peritoneal dialysis did not affect renin substrate in normal rats, but increased it in uninephrectomized rats and in binephrectomized rats The post binephrectomy increase in substrate was markedly more pronounced when both kidneys were removed simultaneously than when nephrectomy was performed in two steps

The increase in plasma renin substrate (angiotensinogen) which follows nephrectomy was first demonstrated by *Leloir et al* (1940) and it has since been found by many investigators (for literature see *Page & McCubbin* 1968) The increase has been assumed to be caused by lack of the normal renin secretion (*Munoz et al* 1940), but recent studies have shown that it is independent of the pre-operative renal and plasma renin (*Bing & Poulsen* 1970) The postnephrectomy increase in angiotensinogen must therefore be due either to loss of some internal renal factor other than renin or to loss of some external renal function

The aim of the present study is to elucidate the last of these two possibilities For this purpose the postnephrectomy increase in renin substrate was studied first in nephrectomized rats in which the blood urea was kept nearly normal by peritoneal dialysis, and thereafter in normal rats with either a vesico-venous anastomosis or continuous intravenous infusion of urine in about the same amount, as that excreted Finally some experiments with infusion of solutions containing different constituents of urine were performed

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MATERIAL AND METHODS

I Peritoneal dialysis 36 female albino rats weighing about 200 g (180 230 g) were used The rats had their kidneys translocated to subcutis about one month before the peritoneal dialysis experiments in order to avert leaking out of the dialysis fluid through the nephrectomy incisions In some animals unilateral nephrectomy was performed simultaneously with translocation of the other kidney

The peritoneal dialysis was performed by means of two catheters an inlet catheter which was a

simple tube, and an outlet catheter consisting of an inner polythene tube (P P 240) with 14 perforations and placed in a 33 mm long outer plexi glass tube (outer diameter 6 mm inner diameter 3 3 mm) with 32 perforations, each of which had a diameter of 1 5 mm The incisions in the abdominal wall were closed tightly around the catheters by means of purse string sutures The prewarmed dialysis fluid was infused and removed by two 50 ml syrings which were coupled so that the volumes of fluid infused and removed were identical, the constancy being checked by continuous weighing of the rat which was placed on a balance Before the replacement of fluid was started, 15 ml of the fluid was injected intraperitoneally, allowing an i p pool of about this volume The speed of the renewal was about 50 ml dialysis fluid per 22 min the total length of the dialysis being 7 hours The rats were prepared for the dialysis experiments by pretreatment with penicillin, anaesthesia with about 25 mg amytal insertion of catheters in the trachea and in the jugular vein (to maintain the anaesthesia) Both the previously uninephrectomized rats and the non nephrectomized rats were classified into 4 groups 1) untreated normal 2) untreated bilaterally nephrectomized, 3) peritoneal dialysed normal, and 4) peritoneal dialysed bilaterally nephrectomized rats

II Vesico venous anastomosis 56 female S P F Wistar rats weighing 200–300 g were anaesthetized with 7–10 mg amytal plus ether Urethra was ligated and a vesico venous anastomosis was formed by joining a polythene tube 90 catheter placed in the urinary bladder with a tube 50 catheter placed in the left jugular vein from where it was drawn subcutaneously around the neck and further along e midline of the front of the thorax and the abdomen Other rats were binephrectomized or sham operated

The duration of the experiments was 7 or 24 hours In the 7 hours experiments blood samples were taken 1) 24 hours before the operation and 2) at the end of the experiment during which the animals were kept anaesthetized In the 24 hours experiments blood samples were taken 1) just before the operation and 2) at the end of the experiment during which the animals were awake apart from two to three hours after the operation The flow through the anastomosis was estimated at the start during and at the end of the experiments by injection of about 0 05 ml of a lissamin green solution with determination of its passage or by direct measurement of the flow

III Continuous intravenous infusion of urine or solutions of some urine constituents Male S P F Wistar rats weighing about 235 g and female rats weighing about 175 g were pretreated as the rats in group II with penicillin anaesthesia insertion of catheters in trachea and jugular vein Blood samples were taken 24 hours before and at the end of the 7 hours experiments The urine, which was centrifuged before injection was either the animals' own, which was collected and injected with about one hour's delay, or a centrifuged sample of a urine pool collected from either male or female rats The pools consisted of urine which was voided when the rats were taken out of their cages The collected samples were frozen to about –20°C shortly after collection No significant difference between the results of experiments using the different urine samples was found In the rats which were infused with their own urine, the volume of excreted urine was increasing with time, being about 0 3 ml in the first hour and increasing to 0 45, 0 55, 0 7, 0 85, 1 0 and 1 15 ml per hour in the following six hours These doses were therefore used also for infusion of donor urine in experiments on female rats weighing about 175 g and were increased corresponding to the higher weight in the male rats The same doses were used for infusion of solutions of 1) 0 9 per cent NaCl 2) 5 5 per cent glucose or 3) a mixture of 230 mg KCl, 152 mg NaCl and 7 3 g urea per litre corresponding to the values found in one of the urine pools When 9 per cent sodium chloride was infused, only one tenth of these doses were given

Blood samples were at once placed at 4°C using 50 µl of a 6 per cent sodium citrate per ml blood as anticoagulans

Plasma urea was determined spectrophotometrically by the method of *Fawcett & Scott* (1960) and *plasma renin substrate* by radio immuno assay for angiotensin II by the method of *Poulsen* (1969) or in some experiments with a slight modification of this method omitting the converting enzyme preparation adding EDTA and using radio-immuno-assay for angiotensin *I Renal renin* was determined by bio-assay of extracts using the standard of dr Haas The *haematocrit* determinations were performed with an Adams autocrit

RESULTS

I Peritoneal Dialysis

As the results of these experiments were found to differ in rats not previously operated and rats nephrectomized about one month previously, these two groups (A and B) have been separated both in the text in Table 1, and in Figs 1 and 2 Each of the two groups are again divided into 1) untreated controls 2) untreated nephrectomized 3) dialysed normal and 4) dialysed nephrectomized rats

A Previously untreated rats (marked Δ in Fig 1 and 2) The plasma *urea* concentra

TABLE 1 *The Left Half of the Table Shows the Plasma Urea (in mg %) and the Right Half the* Renin Substrate *(in ng Angiotensin) in* Untreated *and* Dialysed *Controls or Nephrectomized Rats*

	Urea				Substrate			
	Untreated		Dialysed		Untreated		Dialysed	
	Control	Nephrectomy	Control	Nephrectomy	Control	Nephrectomy	Control	Nephrectomy
A Previously untreated rats								
Before experiment (Range)	*41* (36–47)	*45* (41–50)	*45* (36–53)	*48* (39–61)	*390* (278–510)	*404* (318–500)	*361* (302–394)	*364* (304–436)
After experiment (Range)	*45* (33–55)	*101* (86–116)	*42* (37–46)	*68* (55–95)	*486* (398–560)	*1038* (874–1463)	*306* (180–416)	*1491* (1034–2607)
B. 1 month previously uninephrectomized rats								
Before experiment (Range)	*59* (54–65)	*59* (55–66)	*60* (58–63)	*55* (50–63)	*326* (236–456)	*324* (270–398)	*334* (276–420)	*304* (254–342)
After experiment (Range)	*42* (35–48)	*111* (100–119)	*39* (36–42)	*64* (45–92)	*365* (294–432)	*455* (432–480)	*649* (565–789)	*1037* (913–1216)

Each value is the mean of values from 4 to 6 rats the values being obtained before and after the 7 hours experiments One part (A) was performed on previously untreated rats while the other (B) was performed on rats uninephrectomized about 1 month previously The relation between the values obtained in each of the rats before and after the experiment is given in Fig 1 and 2

simple tube, and an outlet catheter consisting of an inner polythene tube (P P 240) with 14 perforations and placed in a 33 mm long outer plexiglass tube (outer diameter 6 mm inner diameter 3 3 mm) with 32 perforations, each of which had a diameter of 1 5 mm The incisions in the abdominal wall were closed tightly around the catheters by means of purse string sutures The prewarmed dialysis fluid was infused and removed by two 50 ml syrings which were coupled so that the volumes of fluid infused and removed were identical, the constancy being checked by continuous weighing of the rat which was placed on a balance Before the replacement of fluid was started, 15 ml of the fluid was injected intra peritoneally, allowing an i p pool of about this volume The speed of the renewal was about 50 ml dialysis fluid per 22 min, the total length of the dialysis being 7 hours The rats were prepared for the dialysis experiments by pretreatment with penicillin, anaesthesia with about 25 mg amytal insertion of catheters in the trachea and in the jugular vein (to maintain the anaesthesia) Both the previously uninephrectomized rats and the non nephrectomized rats were classified into 4 groups 1) untreated normal, 2) untreated bi laterally nephrectomized, 3) peritoneal dialysed normal and 4) peritoneal dialysed bilaterally nephrectomized rats

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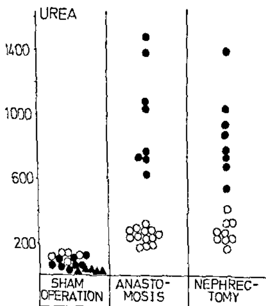


Fig 3 The *changes in plasma urea* during the experiments on *sham operated, vesico venous anastomosed* and *nephrectomized* rats are expressed as in Fig 1 The figure shows the results of 7 hours (marked ○) and 24 hours experiments (marked ●) In the group of sham operated ○ and ● marks values from sham nephrectomized, while the mark ▲ indicates that the rats were sham anastomosed including subcutaneous application of catheters and opening and ligation of the top of the bladder

those in group A (marked △), as seen in Fig 1

Renin substrate was about 330, and thus a little lower than that in previously untreated rats The changes after the experiments were so far similar to those found in rats not previously operated, as there was a markedly higher increase in the dialysed than in the untreated, (in two steps) binephrectomized rats (Fig 2) But the results differ in two ways from those of group A the first difference being that the increase in renin substrate both in untreated and dialysed rats is smaller when the binephrectomy is performed in two steps than when performed in one The second difference is found in the effect of dialysis which in normal rats caused a slight *decrease*, but in uninephrectomized a marked *increase* in renin substrate

II Vesico-Venous Anastomosis

In all rats with vesico-venous anastomosis there was a significant increase in plasma *urea* as shown in Fig 3, in which the values found at the end of the experiments are given in per cent of those found before the operation While the values were about the same before and after the operation in the sham operated animals, those of the rats with vesico venous anastomosis or nephrectomy were about 200 to 300 per cent in the 7 hours' experiments and about 600 to 1500 per cent of the normal values in the 24 hours experiments the degree of urea retention being about the same in the rats with vesico venous anastomosis and in the nephrectomized

The relation between the *renin substrate* concentration before and at the end of the experiments is shown in Fig 4, according to

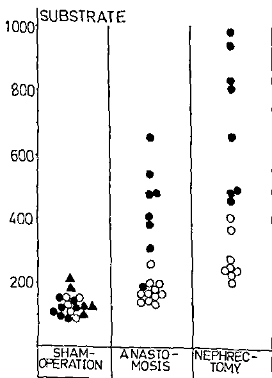


Fig 4 The *percentage changes in renin substrate* during the experiments on sham operated vesico venous anastomosed and nephrectomized rats are expressed and marked as the changes in urea in Fig 3

which the values after 7 hours vesico venous anastomosis only differed a little from the slightly increased values about 150 per cent seen in the sham operated while the 24 hours' experiments resulted in a marked increase up to values between 300 and 700 The values of the nephrectomized rats are however, markedly higher, being about 200 to 400 per cent of the start values in the 7 hours' experiments and about 500 to 1000 per cent in the 24 hours experiments The reason why the changes in renin substrate are stated as the percentage increase rather than in the substrate values in ng angiotensin is that the experiments were performed both on male and female rats in which the renin substrate concentrations are different although the percentage increase is about the same after the different experiments Thus the mean of normal rats was found to be 644 (range 513 777) in 10 males and 344 (range 231–436) in 11 females while that of rats with 7 hours vesico venous anastomosis was 1076 (range 757–1323) in males and 577 (455–760) in females giving about identical mean percentage increases 165 per cent in male and 193 per cent in female rats

The changes in *haematocrit* during the experiments were insignificant in the sham ated animals the mean end values being per cent of the mean start values After ...es co venous anastomosis and after bine phrectomy the changes were identical the percentage being 93 after 7 hours and 89 after 24 hours The differences between the increases in renin substrate in anastomosed and in nephrectomized rats are thus not related to changes in the haematocrit

The weight of the *kidneys* of the rats with vesico venous anastomosis was significantly higher and the renin concentration significantly lower than that of normal rats (Table 2) The resulting renin content of one kidney is a little lower (mean about 65 per cent) in the rats with vesico venous anastomosis

The *flow of urine through the anastomosis* was about 3 10 µl per minute at the start about 100–200 µl after 7 hours and 100–700 µl per minute after 24 hours the maximal urine flow being 700 µl per minute (corresponding to 1 litre per 24 hours) in a rat weighing 240 g The urea concentration in this urine was 420 mg per cent plasma urea being 307 mg per cent and *urea clearance* thus being about 1 ml per minute In two other rats, urea clearances of 0 3 and 0 9 ml per minute were found 24 hours after establishment of the anastomosis When tested with Hema Combistix (Ames) these urines were found not to contain sugar or protein

III Continuous Infusion of Urine or Different Solutions of Urine Constituents

The *haematocrit* was in most rats about 39 (range 33–46) 24 hours before the start of the experiments and there was only a slight fall to about 36 after the different experiments The plasma *urea* was about 40 mg per cent slightly higher in the males than in the females in the Wistar rats (and about 33 in the female rats of the Leo strain which were used in a minority of the experiments) The change in plasma urea is seen in Fig 5 which shows the urea at the end of the 7 hours experiments expressed in per cent of the values at the start It is seen that the values of the controls are about 100 while there is significant increase after nephrectomy (170 to 360) and about the same increases in the animals which received injections of untreated or neutralized *urine* and of a solution containing the same concentrations of urea NaCl as KCl as one of the urine pools used for injection Contrary to these elevated values the values of the animals injected with 0 9 or 9 0 per cent NaCl or glucose solutions or with dialysed urine were mostly lower than those of the controls

The plasma *renin substrate* concentration in the animals used (measured 24 hours before the experiments) was in 14 female rats of the Leo strain 285 (range 202 360) in 42 female rats of the Wistar strain 323 (range 187–502) and in 22 male Wistar rats 479 (range 249–795) These differences did *not* influence the relative changes found during the experiments

TABLE 2 Renal Weight *Is Higher and* Renin Concentration *Is Lower in Rats with Vesico Venous Anastomosis Than in Controls The Renin Content in One Kidney in the Rats with Anastomosis Is Somewhat Lower Than That in the Controls*

Rat	Treatment	Body weight in g	Weight of 1 kidney	Renin units/g	Renin units/1 kidney
1	24 hours	200	0 99	2 4	2 4
2	Vesico-venous	700	1 00	1 9	1 9
3	anastomosis	202	0 98	3 2	3 1
4		200	1 03	2 4	2 5
5		195	0 62	5 6	3 5
6		212	0 71	5 6	4 0
7		207	0 87	7 4	6 4
8	Controls	217	0 86	2 8	2 4
9		200	0 81	4 2	3 4
10		215	0 93	4 5	4 2

The results of the changes in renin substrate after the different 7 hours experiments are shown in Fig 6 In the *controls* the renin substrate was *only little changed during the* 7 hours In the *binephrectomized rats* there was a significant increase up to 225 to 375 per cent of the start values In 14 out of 18 rats which were continuously infused with *urine* there was a marked increase to 180 to 300 per cent of the start values, while the values of the 4 other rats were either in the normal range or only slightly increased Although there is an overlapping of the values in the nephrectomized and the urine infused rats the mean of the nephrectomized rats is significantly higher than that of the rats which received urine infusion The values of the rats injected with either *neutralized or dialysed urine* were partly in the normal range and partly significantly elevated Most of the rats injected with *sodium chloride* solutions had values in the normal range this being the case in all rats which received the 9 per cent NaCl solution But some of the rats (mostly those belonging to the Leo strain) which were injected with 0 9 per cent NaCl solution had markedly increased values ranging from 190 to 310 per cent of the start values The animals which received injections either of the mixture of *NaCl KCl and urea* in a concentration corresponding to a urine pool or of *glucose* had values in the upper half of the range found in the controls

DISCUSSION

1 Cause of Post binephrectomy Increase in Renin Substrate

With the aim of elucidating *whether the post binephrectomy increase in renin substrate is caused by ceased urine excretion* the present study contains 1) studies of binephrectomized rats in which retention of urine constituents was reduced by means of peritoneal dialysis and 2) studies of *non nephrectomized rats* in which a retention of urine constituents was obtained either by vesico-venous anastomosis or by infusion of urine The effectivity of these procedures was measured by determination of plasma urea showing that this urine constituent was in any case significantly lowered and close to normal values in dialysed nephrectomized rats and that it was increased to about the same high values as those in binephrectomized rats both in vesico venous anastomosed and in urine infused non nephrectomized rats

The results of the different experiments do not give an unambiguous answer to the question whether postnephrectomy increase in renin substrate is wholly due to ceased urine excretion The experiments on *dialysed*

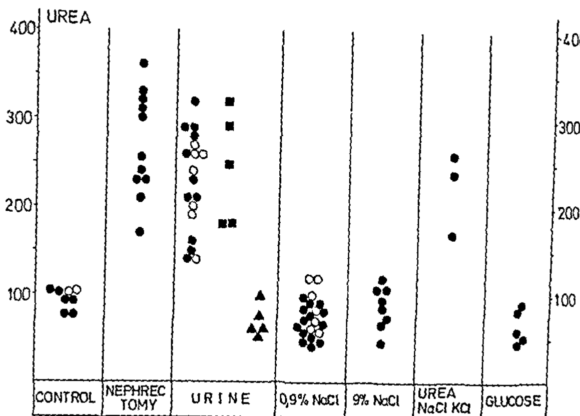


Fig 5 The *percentage changes in plasma urea* during the 7 hours *experiments on controls ne phrectomi ed* and *rats infused with urine or solutions of urine constituents* are expressed as in Fig 3 Experiments on Leo rats are marked ○ those on Wistar rats ● except those on the Wistar rats which received neutralized (■) or dialysed (▲) urine

rats were unsuited for a ...on of the question as it was found that they had *higher* renin substrate than un treated nephrectomized (Fig 2) In rats with *vesico venous anastomosis* there was both in the 7 hours and more pronounced in the 24 hours experiments increases in renin sub strate but in both groups the increases were on an average less pronounced than in the nephrectomized rats (Fig 4) About the same results were found in *urine infused* non nephrectomized rats which in most cases showed increases in renin substrate which however on the whole were less pronounced than those in the nephrectomized rats (Fig 6) The results are here even more com plicated as some rats infused with 0 9 per cent sodium chloride solution had the same increased values as the urine infused animals This was especially the case with rats of the Leo strain probably because solutions con taining water distilled by means of a resin were used for these rats while glass distilled sterile water was used for the Wistar rats The experiments with infusion of 1) the same amount of sodium chloride in a ten times higher concentration 2) an urea sodium and potassium chloride solution and 3) a glucose solution (all three in sterile glass distilled water) show that neither of these infusions resulted in increased renin substrate

According to a recently published paper *Blaine et al* (1971) have studied the cause of the postnephrectomy increase in renin sub strate in dogs in a way which is identical with that used in a part of the present *study* They found that renin substrate was markedly elevated in dogs with renal tubular damage involving stopped glomerular filtration while it was only slightly elevated in dogs with

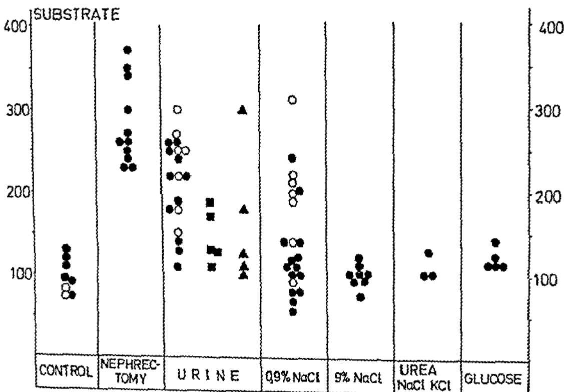


Fig 6 The *percentage changes in renin substrate* during the 7 hours' experiments on *controls, nephrectomized* and rats *infused with urine or solutions of urine constituents* are expressed as in Fig 3 Symbols as in Fig 5

uretero-vena cava shunts They concluded that renin substrate may in part, be controlled by changes in glomerular filtration or alterations in renal tubular function

The results of the present study of rats differ from those which *Blaine et al* obtained in dogs, as there was a marked increase in substrate in rats with vesico-venous anastomosis as well as in urine infused rats Although these increases on the whole were less pronounced than those found in nephrectomized rats there was an overlapping of the values and retention of urine substituents can thus be *part of the cause* of the high substrate values found in nephrectomized rats The experiments do not exclude the possibility that urine-injection in form of an anastomosis or an infusion in some way hampers the response in substrate to the increase in plasma of some urine-constituents If so, the ceased urine excretion can be the *whole cause* of the post-nephrectomy increase in substrate A third possibility is that the increases in renin substrate found in nephrectomized rats and in vesico-venous anastomosed (or urine-infused) rats are due to quite *different mechanisms*, apparently in the same way as the increases found in nephrectomized (or ureterligated) and hypoxic rats (*Bing & Poulsen* 1969) and in nephrectomized and estradiol treated rats (*Nasjletti et al* 1969) If so, the postnephrectomy increase in substrate is due either to loss of some internal renal factor or caused through some effect on the circulation induced by ligation of the renal arteries

2 *Effect of Peritoneal Dialysis on Renin Substrate*

Peritoneal dialysis, which in normal rats was without influence produced a marked

increase in plasma renin substrate in rats uninephrectomized 1 month previously, and still higher values in binephrectomized rats in which the values were markedly higher than in untreated nephrectomized rats (Fig 2 and Table 1) Such effect of peritoneal dialysis on renin substrate may partly or wholly be the cause of the increased plasma renin activity found by *Takeuchi et al* (1970) after peritoneal dialysis of patients with chronic renal failure

3 *Reduced Post Binephrectomy Increase in Renin Substrate in Previously Uninephrectomized Rats*

In some experiments binephrectomy was performed in two steps, one kidney being removed about 1 month before the other, while in other experiments the two kidneys were removed simultaneously These different forms of binephrectomy resulted in different increases in renin substrate, the increases being markedly higher when both kidneys were removed in one step than when first one and later the other kidney were removed (Fig 2) This difference, which was found in experiments of 7 hours' duration, has also been found in many experiments in which the increase in renin substrate was determined hours after removal of the kidneys in one two steps (*Bing & Jorgensen*, in press)

Supported by a grant from *King Christian X Foundation*

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REDUCED POST-BINEPHRECTOMY INCREASE IN RENIN SUBSTRATE IN PREVIOUSLY UNINEPHRECTOMIZED RATS

Including Studies of Uni-Ureter Ligated Rats, Hypoxia or Oestradiol Pretreated Rats, and of Renal Hypertensive Rats

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While renin substrate is increased to values of about 2000–4000 ng 24 hours after simultaneous binephrectomy, the values obtained in *previously uninephrectomized* or *unilaterally ureter ligated rats* are only about 50 to 75 per cent as high (about 1000 2500 ng) 24 hours after removal of the remaining kidney This decreased reaction is found as early as 30 minutes after uninephrectomy and is still found well over a month later If the operations (binephrectomy or removal of remaining kidney) are preceded by 17 hours *hypoxia*, the values in rats whether binephrectomized in one step or in two steps are equally low (600–1700), while pretreatment with *oestradiol* in both groups results in about equally extremely high values (3500–6000) Previously uninephrectomized *renal hypertensive rats* show the same decreased response as normal rats if the untouched kidney is removed before the clipped, but if the clipped kidney is removed before the untouched, the values are as high as those found when both kidneys are removed at the same time The effect of previous uninephrectomy seems neither related to the decrease in renal renin or to the retention of urinary constituents caused by the operation

It is well known that *binephrectomy* of rats is followed by an increase in renin substrate (angiotensinogen) to values about 5 to 12 times higher than those found in normal rats (for literature see *Page* and *McCubbin* 1968) It has further been shown that *uninephrectomy* results in changes into values which after 24 hours are 2 to 3 times as high (*Bing* and *Poulsen* 1970) but after 4 weeks are only about 25 per cent increased (*Carretero* and *Gross* 1967) In studies of rats in which *first one* kidney was removed and about one month later the remaining kidney was removed, it has recently been shown that the post binephrectomy increase in substrate is markedly smaller when the binephrectomy is performed in two steps, than when both kidneys are removed simultaneously (*Bing* and *Jørgensen* 1971, in press) The aim of the present paper was to obtain further information about the changes in renin substrate in uni and binephrectomized rats, including both normal rats and a limited number of renal hypertensive animals The paper also contains some experiments in which the effect of binephrectomy was studied in uni ureterligated rats and in some animals which were treated with oestradiol or made hypoxic in the interval between removal of the first and the second kidney

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MATERIALS AND METHODS

Most of the experiments were performed on female SPF Wistar rats some on male rats of the same strain and some on female Sprague Dawley black headed rats The rats were maintained on an *ad libitum* diet of laboratory chow and water Nephrectomy and shamoperation were performed on ether anaesthetized penicillin pretreated animals Blood samples were taken from the carotic or the femoral artery and at once placed at about 0–4° C using 50 µl of a 6 per cent sodium citrate per ml blood as anticoagulant Plasma *renin substrate* was determined by radioimmunoassay for angiotensin I using a slight modification of the method of Poulsen (1969) omitting the converting enzyme preparation and adding EDTA Plasma *urea* was determined spectrophotometrically and the *haematocrit* with an Adams autocrit

Renal hypertension was induced by partial clamping of one renal artery with a silver clip (*Wilson* and *Byron* 1939) *Hypoxia* was obtained by keeping the rats for 17 hours in a vacuum tank in which the air was renewed continuously and the pressure kept constant at 300 mm Hg by help of a high vacuum pump The *oestradiol* preparation used was an 0 25 per cent solution of oestradiol benzoate in peanut oil containing 2 5 mg per ml of which 0 3 ml was given intraperitoneally at four days and 0 5 ml on the fifth day in the week for about 10 days

RESULTS

1 *Effects of Previous Uni Nephrectomy on Post Binephrectomy Increase in Renin Substrate in Normal Rats*

Renin substrate was determined *before the experiments* in 52 female Wistar rats weighing about 180 g and in 9 females weighing about 245 g The mean value was 385 ng per ml (range 270–560) 90 per cent of the values ranging between 300 and 475 ng In the 9 older females, the mean value was 452 ng (range 400–520) Renin substrate was not determined before the experiments in the 11 male rats included in this study (mean body weight 306 g (range 220–340))

The effects of shamoperation and of uninephrectomy on renin substrate are seen in Fig 1 which shows the percentage changes from the individual pre operative values found at intervals of from 1 hour to 3 weeks after the operations While the renin sub

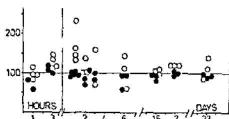


Fig 1 The renin substrate values of shamoperated (marked ●) and uninephrectomized (marked O) rats at various times in hours and days after operations are given in per cent of the individual pre operative values

strate of the *shamoperated* animals is unchanged or slightly decreased, the values of those *uninephrectomized* are increased up to between 140 and 230 per cent of the pre operative values 24 hours after uninephrectomy, but only slightly increased up to about 125 per cent (range 75–160 per cent) both after intervals of 3 hours and of 2 to 23 days after uninephrectomy in accordance with the increase after 4 weeks found by *Carretero* and *Gross* (1967)

The effects on the renin substrate found 24 hours after *binephrectomy* are seen in Fig 2 which shows that the postbinephrectomy increase is smaller in previously uninepréctomized than in previously shamoperated rats This difference is *not* found if there is only 15 minutes between the two operations but it is marked even when there is only 30 minutes between uni and binephrectomy and it is found in all experiments in which the time between uninephrectomy and removal of the second kidney is longer *Most of the* values of binephrectomized female rats range from about 1000 to 2000 ng per ml in the previously uninephrectomized rats and from about 1800 to well over 4000 ng in those previously shamoperated in which both kidneys were removed simultaneously In the female rats which were binephrectomized 1 to 7 hours after the uninephrectomy and in the male rats the values were on an average about 50 per cent higher but still with a marked difference between previosuly sham operated and uninephrectomized animals Such differences between the values obtained

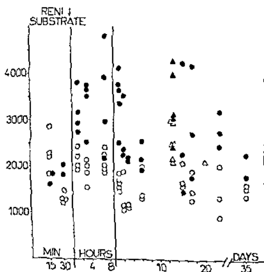


Fig 2 The different increases in renin substrate (ng/ml) found 24 hours after binephrectomy of previously shamoperated (marked ●) and uninephrectomized (marked ○) female rats at various times in minutes, hours and days after sham operation or uninephrectomy Similar findings in a few male rats are marked ▲ and △

in groups of rats binephrectomized at different times after uninephrectomy or sham operation are not due to analytical errors This was shown by inclusion of a "standard plasma"[3] in the daily analyses the variations in the standard being less than ± 10 per cent But they can be due to unknown environmental factors, as the experiments included in Fig 2 were performed during a period of about ten months while the rats belonging to an individual group, corresponding to a special time after the first operation and thus placed vertically over each other in fig 2 were binephrectomized the same day

The mean plasma urea was 41 mg per cent (range 18–60) both in *untreated* and *sham operated female rats* The values were about 18 per cent increased 1 and 3 hours after *uninephrectomy* and about 24 *per cent in*creased if intervals were longer 24 hours after *binephrectomy* the mean urea values were identical (*mean 300* mg per cent) in previously uninephrectomized and in previously shamoperated rats

The mean *haematocrit* was about 42 per cent (range 37–45) both in *untreated, shamoperated* and *uninephrectomized* rats Twenty four hours after binephrectomy there was no difference between the values of previously uninephrectomized and previously shamoperated animals, which values were identically decreased to about 29 in the rats binephrectomized 1 and 3 hours after the first operation and to about 36 if intervals between operations were longer

2 *Effect of Previous Ligation of One Ureter on Post-Binephrectomy Increase in Renin Substrate*

In studies of 6 female Wistar rats uni ureter-ligated one to three days before and of 4 animals shamoperated (weight about 200 g), (Fig 3A), uni ureterligation was found to have the same effect as uni nephrectomy The effects on *plasma urea* and *haematocrit* were also similar to those found in the uni nephrectomized animals

3 *Effects of Hypoxia on the Post Binephrectomy Increase in Renin Substrate of Previously Uninephrectomized or Sham operated Rats*

The effect of pretreatment wih a 17 hours' period of hypoxia at 300 mm Hg immediately before binephrectomy was studied in two previously uninephrectomized and two sham operated female Sprague Dawley rats (weight about 250 g) and in two uninephrectomized and two shamoperated female Wistar rats (weight about 185 g) Fig 3B shows the renin substrate values 1) 2 months after the first operation, 2) at the end of 17 hours hypoxia (just before binephrectomy), and 3) 24 hours after binephrectomy After hypoxia, most values are increased to 800–1200 ng and after subsequent binephrectomy, most values rise to 1200–1700 ng which is a little lower than the average values found after removal of the second kidney of previously uninephrectomized (non hypoxic) rats It is seen that the marked difference between the values of non hypoxic uninephrectomized and

shamoperated rats is lost when the rats are made hypoxic before binephrectomy

Though they had access to food and water the rats lost about 13 per cent in *body weight* during the hypoxic period In some rats the *plasma urea* fell by about 30 per cent, in others it was unchanged or increased after hypoxia After binephrectomy, the urea values were about 300–350 mg per cent The *haematocrit* was not significantly changed after hypoxia, but as usual lowered after binephrectomy

4 *Effects of Oestradiol on the Post-Binephrectomy Increase in Renin Substrate of Previously Uninephrectomized or Shamoperated Rats*

The effect on renin substrate of about 10 days' treatment with oestradiol was studied in 8 previously uninephrectomized and 8 shamoperated Wistar, Sprague Dawley or blackheaded rats (weight 250–300 g) Four of the rats received 0 5 mg oestradiol benzoate *subcutaneously* per day, which did not influence the renin substrate significantly, and after subsequent binephrectomy values were as usual found to be lower in the previously uninephrectomized animals (2420 and 2690) than in those in which both kidneys were ...ved simultaneously (3488 and 4360) ...ontrary to this, 10 days of treatment with 0 75 mg oestradiol benzoate *intraperitoneally* per day resulted in a marked increase in renin substrate, up to about 1400 ng, in animals uninephrectomized 2 months previously as well as in unoperated rats, as seen in Fig 3C The figure further shows that, when these rats were binephrectomized, there was about the same increases to values of about 4500 ng (range 2500–6100) both in the rats which were nephrectomized in two steps, and in those in which both kidneys were removed simultaneously There was no difference between the results obtained in the different strains of rats

During the treatment with oestradiol, *plasma urea* was in some rats found to be increased, in others decreased The different

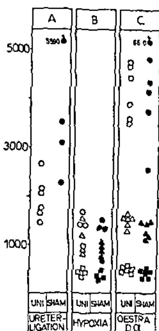


Fig 3 *Fig 3A* shows the differently increased values for renin substrate 24 hours after binephrectomy of previously *uni ureter ligated* (marked *UNI*) and shamoperated (marked *SHAM*) rats *Fig 3B* and *3C* show similar studies of rats which were pretreated with *hypoxia* or oestradiol during intervals between removal of one kidney (*UNI*) or shamoperation and binephrectomy These figures show the preoperative values (□ and ■), the values obtained after hypoxia or oestradiol treatment (△ and ▲) just before binephrectomy and the values obtained 24 hours after binephrectomy (○ and ●)

response was *not* related to the changes in substrate or in haematocrit, and was found both in uninephrectomized and shamoperated animals

5 *Effects on Renin Substrate of Removal of Either the Clipped or the Untouched Kidney and of Secondary Removal of the Remaining Kidney in Renal Hypertensive Rats*

Nine renal hypertensive black headed female rats were uninephrectomized 7 to 13 months after application of a clip on the renal artery In five of these, in which *the clipped kidney was removed first*, the renin substrate was only little increased 3 days after uninephrectomy (△ Fig 4A) Twenty

A | B | C | D

5000

3000

1000

CLIPPED FIRST | UNTOUCH FIRST | SHAM OP

RENAL HYPERTENSIVE | NORMAL

Fig 4 The renin substrate values of *renal hypertensive rats* in which the clipped kidney was removed 3 days before the untouched are recorded in *fig 4A*, while *fig 4B* shows the values in rats in which the untouched kidney was removed first. The values in hypertensive rats in which the clipped and the untouched kidney were removed at the same time are recorded in *fig 4C*. *fig 4D* shows the values in normal rats in which the binephrectomy was performed in one or in two steps. Symbols as in fig 3

four hours after subsequent removal of the second (untouched) kidney, he values of four of these were as high (range 2100 to 5500 ng) as those found after simultaneous removal of both kidneys in *normal* rats while one value was only about 1320 (○ Fig 4A). In the four other renal hypertensive rats in which *the untouched kidney was removed first*, the results obtained after uni and binephrectomy were quite different. Three days after removal of the untouched kidney, the renin substrate values were 2 to 3 times increased (△ Fig 4B), and 24 hours after subsequent removal of the other (clipped) kidney, the values were at the same *relative* low level (○ Fig 4B) as those found in *normal* rats after removal of the second kidney after previous uninephrectomy (○ Fig 4D).

The *relative weight of the two kidneys* of the renal hypertensive rats was changed in the usual way, the weight of the clipped kidney being only 32 per cent (range 19–45) of the weight of both kidneys.

The *plasma urea*, which in normal rats was about 41 mg per cent (range 18–60), was about 52 (range 30–59) in the hypertensive rats. 3 days after removal of the clipped kidney only one out of five rats had significantly increased urea, while a marked increase to 69–378 mg per cent was found after removal of the untouched kidney. Twenty-four hours after binephrectomy, which in normal rats resulted in values of about 300 mg per cent, somewhat higher values of about 360 mg per cent (range 280–580) were found. The changes in *haematocrit* values of untouched, uni- and binephrectomized hypertensive rats, did not differ from those found in similarly treated normal rats.

DISCUSSION

1 *Different Response to Binephrectomy in Differently Pretreated Rats*

The present study has confirmed our previous finding (1971) of a *reduced post binephrectomy increase in renin substrate in previously uninephrectomized rats* (Fig 2), the substrate values of previously uninephrectomized being about 50 to 75 per cent of those of rats whose kidneys both were removed at the same time. This reduction was found in both male and female Wistar, Sprague Dawley and black headed rats. The same reduction was found after *unilateral ureter ligation* (Fig 3A) and after *removal of the untouched kidney of renal hypertensive rats* (Fig 4B). Contrary to this, the renin substrate was found to be equally increased in simultaneously binephrectomized rats and in rats in which first one and later the other kidney were removed if the animals before binephrectomy were made *hypoxic* (Fig 3B) or treated with *oestradiol* (Fig 3C), and if *the clipped kidney was removed before the untouched in renal hypertensive rats* (fig 4A). It applies to these three groups of animals that values in hypoxic animals were

much lower, in oestradiol treated animals higher, and in renal hypertensive animals *values were found to be about* the same as those found after binephrectomy of normal rats

2 *Influence of Renal and Plasma Renin*

The reason why the increase in renin substrate is of different degrees in arenal rats is as obscure as the reason why such increase in substrate occurs at all after binephrectomy The obvious reason, *i e* that renin substrate is increased in binephrectomized animals because of removal of the site of renin formation as first proposed by *Munoz* et al (1940), was invalidated by studies according to which the increase in substrate is equally high in rats with very low preoperative renal and plasma renin and in normal rats (*Bing* and *Poulsen*, 1970) This finding was con firmed in the present study where removal of the renin poor, untouched kidney in hyper- tensive rats (after previous removal of the clipped) was found to result in an increase in renin substrate (○ Fig 4A) of the same order as that found after simultaneously removal of both kidneys of a normal rat (● Fig 4D and Fig 2) A further sign of the *lack of relation between the removed amount of renal renin and the increase in substrate* is the finding [illegible]t removal of the renin rich clipped kidney [illegible] previous removal of the untouched) results in the same reduced increase in sub strate (○ Fig 4B) as that found after re moval of the remaining kidney of a previously uninephrectomized normal rat (○ Fig 4D and Fig 2), in which the kidney contains much less renin than the clipped kidney (*Gross* 1971)

3 *Influence of Decreased Renal Function*

In studies of the cause of post binephrec tomy increase in renin substrate, *Blaine et al* (1971) found that substrate was only slightly elevated in dogs with ureterovenous shunts and concluded that the increase is not due to ureaemia Such unambiguous conclusion could not be drawn from the results obtained in our studies (1971) of rats, according to which there is a marked increase in substrate in animals with vesico venous anastomosis or continuous urine infusion although increases did not reach the values found after bi nephrectomy

After uninephrectomy the renal function decreases with increased plasma urea, as also found in this study *That the decreased post binephrectomy increase of renin substrate in previously uninephrectomized rats is due to a retention of some substance which inhibits the substrate formation is made improbable* by the finding of a decreased response already 30 minutes after uninephrectomy If the small retention found at this early time should be active, the more pronounced retention following simultaneous removal of both kidneys should be at least equally active

4 *Influence of Discontinued Excretion in the Urine, Catabolism in the Tubules or Release of Inhibitor (Relation between Renin Substrate and Other Renal Dependent Plasma Proteins)*

Besides renin substrate, other plasma proteins are increased after uni and bi nephrectomy As most of these have small molecular weights ranging between 15 000 to 25 000, like alkaline ribonuclease (*Rabino witch* and *Dohl* (1956) and *Royce* (1967)) and the α globulin studied by *Royce* (1968), Bence Jones protein and isolated light chains (*Wochner et al 1967)* and muramidase (*Keeler* 1970), it has been believed that the effect of nephrectomy is due to the failure of excretion in the urine and (or) catabolism in the tubules It cannot be excluded how ever, that the increased concentrations of these proteins after binephrectomy are caused by loss of a *renal inhibitor* of their synthesis Renin substrate differs from these proteins in that it has a molecular weight of 57 000 (*Skeggs et al* 1964) in the small (if any) excretion in the urine and *in the so* far unknown degree of catabolism in the tubules The existence of a renal inhibitor is equally unproven whether the synthesis of renin substrate or the synthesis of the other renal dependent proteins are concerned If a

renal inhibitor of renin substrate synthesis exists it can explain both the post binephrectomy increase in substrate and the small increase after uninephrectomy, but not the finding of the reduced post binephrectomy increase after uninephrectomy

5 *Effect of Hypoxia*

Hypoxia has been shown to provoke an increase in renin substrate which differs both in progress with time and in magnitude from that seen after binephrectomy The substrate of hypoxic rats reaches a maximum after about 17 hours returns to normal values after about 40 hours and remains there even when hypoxia is continued If hypoxia is induced in recently binephrectomized rats a summation of the two effects is *obtained* while binephrectomy performed more than 40 hours after the start of hypoxia results in the same increase in substrate as that seen in non hypoxic rats (*Bing* and *Poulsen* 1969)

The present finding that binephrectomy performed at the time of the maximal effect of hypoxia will result in an equally reduced increase in substrate in previously uninephrectomized and in shamoperated rats (fig 3B) cannot be due to exhaustion of substrate as plasma and renal renin levels are not increased at this stage of hypoxia (*Gould and Goodman* (1970))

6 *Effect of Oestradiol*

Oestrogens are known to bring about an increase in renin substrate (*Helmer* and *Griffith* 1952) which in binephrectomized rats is superimposed on that elicited by nephrectomy (*Nasjletti* et al, 1969) The present study shows that oestradiol causes about the same increase in previously uninephrectomized (△ fig 3C) and in sham operated (▲ fig 3C) rats and that the values 24 hours after binephrectomy of the sham operated animals agree with findings in studies by *Nasjletti et al* in giving a summation of the two effects (● fig 3C) The almost equally high values in the previously uninephrectomized rats (○ fig 3C) suggest that these values are about the highest obtainable

Supported by grants from *King Christian X s foundation*

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Acta path microbiol scand Section A 80, 38-46, 1972

SYMPATHETIC INNERVATION OF THE JUXTAGLOMERULAR CELLS OF THE KIDNEY IN RATS WITH RENAL HYPERTENSION

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Unilateral renal artery stenosis was produced in 60 rats Hypertension developed in 46 and was allowed to persist for various lengths of time Nine unoperated rats served as normal controls The adrenergic innervation pattern of the kidneys was examined by the histochemical fluorescence method In the stenosed kidney of the early hypertensive rat there was a reduction and disappearance of the nerve pattern, particularly at the juxta glomerular level Prolongation of the hypertension first led to normalization of the nerve pattern in the stenosed kidney, and later to the appearance of large numbers of intensely fluorescent nerve fibres in this kidney The possibility that the innervation alterations are the result of a denervation of the stenosed kidney followed by a re innervation is discussed but found less probable It is alternatively suggested that the findings reflect a depletion of the adrenergic transmitter due to an angiotensin induced increase in the release of the substance during the developmental phase of the hypertension The findings in the stenosed kidney of late hypertensive rats may either reflect an over loading by adrenergic transmitter or be a non specific feature secondary to the contraction of the kidney parenchyma

It is probable that the production and release of renin from the juxtaglomerular cells of the kidney (the JG cells) can be regulated by various mechanisms (*McCubbin* 1968) Numerous recent data strongly suggest that the activity of the renal sympathetic nervous system is one such mechanism (*Vander* 1965 *Bozovic & Castenfors* 1967, *Wågermark et al* 1968), but it is not known whether this plays any significant role in the pathogenesis of hypertensive states secondary to increased production and liberation of renin

Received 17 v 71

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It is generally accepted that in hypertension due to renal artery stenosis the rise in blood pressure is the result of a stimulation of the JG cells with a consequent increase in their production and liberation of renin The present experiments which have been preliminarily reported earlier (*Ljungqvist* 1970) were undertaken with the aim to investigate the importance of the juxta glomerular sympathetic innervation for this JG cell stimulation

MATERIAL AND METHODS

The material consists of 69 female Sprague Dawley rats weighing between 180 and 200 g at the beginning of the experiments The rats were kept

on tap water *ad libitum* and laboratory chow containing 0 4 per cent sodium chloride The blood pressures were assessed by the tail pletysmographic method with the rats under brief ether anaesthesia Blood pressure measurements were made on three different occasions before the experiments started and at regular intervals thereafter

Nine of the rats were left unoperated (*Group I*) They served as normal controls

On 60 rats unilateral renal artery stenosis was produced through the application of a 0 15 mm wide silver clip on the left renal artery of the anesthetized animal Of these operated rats fourteen remained normotensive (*Group II*) whereas forty six developed hypertension (*Group III*) Hypertension was considered to have developed when the blood pressure became stabilized at a level above 140 mm Hg, provided that this included a rise of minimum 15 per cent of the original blood pressure This blood pressure elevation occurred at highly varying time intervals following the operation – from a couple of days up to between 6 or 7 weeks (*Table 1*)

Since preliminary data suggested that the adrenergic innervation patterns in kidneys from rats with brief periods of hypertension differed from that in kidneys from rats with hypertension of long standing the hypertensive rats were divided into subgroups as follows

Group III A, which consists of twenty nine rats

[illegible] consists of seven rats sac

TABLE 1 *The Table Shows the Intervals in Days from the Production of Left Renal Artery Stenosis to the Death of the Animals of the Various Groups (Int 1) It also Shows the Intervals from the Production of Renal Artery Stenosis to the Appearance of Blood Pressure Elevation in the Various Groups of Hypertensive Rats (Int 2)*

Group	No of rats	Int 1 (days)	Int 2 (days)
II	14	21 ± 16	—
III A	29	17 ± 7	14 ± 6
III B	10	22 ± 8	14 ± 9
III C	7	397 ± 106	27 ± 16

The figures are means ± SD
Group II normotensive rats
Group III A rats with hypertension for less than 1 week
Group III B rats with hypertension for 1 3 weeks
Group III C rats with hypertension for 2 11 months

rificed after hypertensive periods of 2 11 months

The rats of Group II were killed at different intervals following the operation so that they covered the range of intervals from operation to killing of the hypertensive rats in groups III A and B Table 1 shows the mean intervals from operation to death of rats in the various groups, and the mean intervals from operation to the development of hypertension in the rats of group III

At the end of the experimental period of the individual rats, their blood pressure was determined under ether anaesthesia and their kidneys exposed and removed Pieces of tissue from each kidney were rapidly frozen in liquid propane cooled by liquid nitrogen The rats were killed by an overdosage of ether

The pieces of kidney tissue were processed according to the histochemical fluorescence method for the demonstration of biogeneic mono amines (for references see *Corrodi & Jonsson* 1967, *Fuxe et al* 1970) This included freeze drying of the specimens at –30°C (*Olson & Ungerstedt* 1970) after which they were exposed to formaldehyde gas (water content of formaldehyde powder 0 6 per cent) for 1 hour at +80°C Following vacuum embedding of the specimens in paraffin they were serially sectioned mounted in Entellan (Merck) with added Xylol and examined in the fluorescence microscope Some sections of each series were stained with van Gieson s connective tissue stain counterstained with Weigert s elastin

The serial sectioning was done in order to make it possible to examine the entire circumference of a glomerulus to make it certain that the examination included the vascular pole where the juxtaglomerular cells are located The staining of some of the sections in each series was intended for a determination of the histological alterations that were expected to be present in the left kidneys of the operated animals

The rats of group I were used as controls of the method, since it is known that various environmental factors such as air humidity may influence the histochemical fluorescence reaction For this reason pieces of kidney tissue from 2–3 of these normal rats were included in each batch of kidneys from groups II and III

Each section was labelled according to a codified system and was thus examined without knowledge of the clinical picture of the rat

RESULTS

The fluorescence reaction in the kidneys of rats in the various groups is summarized in Tables 2 and 3

Group I All these unoperated, normal controls showed normal intrarenal adrenergic in

TABLE 2 *Adrenergic Innervation Pattern in Left (Stenosed) Kidneys of Hypertensive (Group III) and Normotensive Rats (Group II), and in Left Kidneys of Normal Controls (Group I) In the Rats of Group III Hypertension Had Been Present for Less than 1 Week in Subgroup A 1–3 Weeks in B and 2–11 Months in C*

Pattern	Group I	Group II	Group III		
			A	B	C
Normal	9	6	0	8	7
Reduced	0	3	4	2§	0
Absent	0	5*	25°	0	0

* All kidneys necrotic
° Two kidneys necrotic
§ One kidney necrotic

nervation patterns Thus, yellowish green fluorescent varicose fibres were seen alongside the arteries and pre and postglomerular arterioles including the parts of the arterioles which contain granulated juxtaglomerular cells (Figs 1 and 2) The adrenergic innerva tion of the efferent juxtamedullary arterioles was seen as fluorescent varicose fibres in the vascular bundles of the outer medulla

Group II These rats were normotensive throughout the experimental period, and they gained weight normally At termination of the experiments it was ascertained by careful spection that the stenosing clip was still in position

In five of these rats gross inspection and histological examination showed severe necro sis and atrophy of the left kidney In some of the remaining nine rats areas of necrosis

TABLE 3 *Adrenergic Innervation Pattern in Right (Non Stenosed) Kidneys of Hypertensive (Group III) and Normotensive Rats (Group II) and in Right Kidneys of Normal Controls (Group I) In the Rats of Group III Hypertension Had Been Present for Less than 1 Week in Subgroup A 1–3 Weeks in B and 2 11 Months in C*

Pattern	Group I	Group II	Group III		
			A	B	C
Normal	9	14	13	10	7
Reduced	0	0	15	0	0
Absent	0	0	1	0	0



Fig 1 Fluorescence microphotograph of a normal rat kidney (Group I) showing varicose fibres around two cross sectioned arteries (arrows) The autofluorescence of the elastic lamellae and tubules is normal (C f Fig 5) × 350

and atrophy were encountered but a major portion of the kidney was normal The right kidney was normal in all rats

Fluorescence microscopy showed normal adrenergic innervation patterns of all right kidneys in this group Six of the left kidneys with a moderate or minor degree of atrophy and necrosis also showed a normal nervous pattern whereas three showed a reduced innervation This included a more sparse distribution of fluorescent varicose fibres along the intrarenal arterial tree and a less intense fluorescence reaction than in the other five kidneys (Fig 3) Whether the innervation pattern was normal or reduced nerve filaments were observed to about the same extent in normal and altered tissue in the individual kidney In the five completely



Fig 2 Fluorescence microphotograph of a normal rat kidney (Group I) showing varicose fibres around a small artery (larger arrow) and along an arteriole (smaller arrow) which is branched off from this artery and leads to a glomerulus (dark area in top left corner) (C f Fig 4) × *600*

necrotic left kidneys, however, no varicose fibres were observed

Group III A Gross inspection and histological examination showed wide spread necrosis in two of the left kidneys in this group of rats whereas limited areas of necrosis or no alterations at all were noted in the remaining 27 kidneys All right kidneys were normal

Fluorescence microscopy showed a reduced adrenergic innervation in four left kidneys In the remaining 25 kidneys fluorescent varicose fibres were not observed at the juxtaglomerular level of the vascular tree (Fig 4) Some of these kidneys appeared to be devoid of fibres also along the larger intrarenal arteries (Fig 5) but in most kidneys fluorescent fibres were observed at this level of the vascular tree although they appeared reduced in number and fluorescence intensity (Fig 4) The right kidneys in this group of rats showed a normal pattern in 13 instances whereas the innervation pattern was reduced in 15 and abolished in one

Group III B Gross inspection and histological examination showed wide spread necrosis with atrophy and some fibrosis in only one left kidney in this group of rats The remaining left kidneys were unaltered or displayed a minor degree of atrophy The right kidneys were normal

Fluorescence microscopy showed a normal adrenergic innervation pattern in eight left kidneys and in all right kidneys (Fig 6) The two remaining left kidneys showed a reduced



Fig 3 Fluorescence microphotograph from the stenosed kidney of a normotensive rat (*Group II*) Weakly fluorescent varicose fibres surround the juxtaglomerular part of an afferent arteriole (arrows) Dark area to the left of this arteriole corresponds to the glomerular tuft × *600*

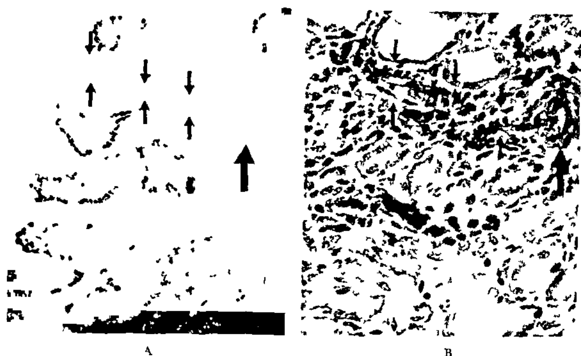


Fig 4 A Fluorescence microphotograph from the stenosed kidney of an early hypertensive rat (Group III A) Weakly fluorescent fibres surround a small artery (larger arrow), whereas the arteriole which is branched off from this artery and runs across the picture towards left (between smaller arrows) is devoid of nerve fibres Dark area at left *corresponds to the glomerular tuft of this ar*teriole (C f Fig 2) × 350

Fig 4 B Neighbouring histological section of the area depicted in Fig 4 A showing the small artery (large arrow) from which the afferent arteriole is branched off and runs (between smaller arrows) to *its glomerular tuft (at left margin of picture) van Gieson elastin × 320*

innervation One of these was the kidney with gross and histological evidence of ne... and atrophy, necrotic areas were ...oid of varicose fibres whereas sparse and weakly fluorescent fibres were encountered alongside the vessels in non necrotic tissue

Group III C In all left kidneys there was severe contraction with histological evidence of fibrosis and tubular atrophy Many glomeruli were occluded and partially or completely fibrotic Large areas of scarring with calcification were seen in some kidneys Usually the most marked histological altera*tions were observed in kidneys from rats with* hypertension of longest standing

The right kidneys also showed evidence of *contraction* although less marked than the left kidneys In the kidneys from rats with hypertension of longest standing numerous glomeruli were occluded fibrosed and hyalinized Lesions of arterial and arteriolar walls were rare and consisted in a minor degree of hyalinization of the vascular walls in some kidneys

Fluorescence microscopy showed numerous intensely fluorescent varicose fibres in the left kidneys The networks of fibres were particularly dense in contracted areas where the fibres accompanied the vessels in their irregular courses through the fibrotic parenchyma (*Fig 7) The right kidneys showed* a more normal distribution of both the vessels and the varicose nerves which is in agree*ment with the much* less marked contraction of the parenchyma

DISCUSSION

In the early phase of hypertension due to unilateral renal artery stenosis an increase *in*



Fig 5 Fluorescence microphotograph from the stenosed kidney of an early hypertensive rat showing a large artery with clearly autofluorescent elastic membranes No varicose fibres are seen around this artery (C f Fig 1) × 350

plasma renin activity and increased degrees of juxtaglomerular cell granulation and renin content of the stenosed kidney will be found (*Tobian* 1966) The non stenosed kidney shows the opposite reaction with a decrease in juxtaglomerular cell granulation and renin content These features are generally interpreted as a stimulation of the juxtaglomerular cells of the stenosed kidney which increase *their production and release of renin* whereas the corresponding functional activities of the juxtaglomerular cells in the opposite kidney are depressed Whether the intrarenal nervous system plays any significant role in these reactions is not known It has been shown however, that electrical stimulation of the renal nerves will result in an increase in plasma renin activity (*Vander* 1965), and that denervation of the kidney will result in a decreased degree of juxtaglomerular cell granulation suggesting a decrease in renin production (*Tobian et al* 1964) Moreover, adrenergic nerve terminals are located in direct relationship to the granulated juxtaglomerular cells (*Wågermark et al* 1968) These data, together, suggest that the sympathetic nervous system may exert a *direct action on the renin producing* juxtaglomerular cells

The finding in the present study of an absent or sparse and weakly visible sympathetic juxtaglomerular innervation in the stenosed kidney of early hypertensive rats (Group III A) and a normal nerve pattern in the kidneys of rats with a slightly more prolonged hypertension (Group III B) appears to be most easily explained on the basis of a surgical denervation of the kidney

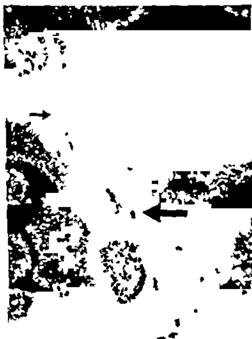


Fig 6 Fluorescence microphotograph from the stenosed kidney of a rat hypertensive throughout 2 weeks (Group III B) Varicose fibres are seen around a transversely sectioned interlobular artery (bottom right) and along an afferent (larger arrow) and efferent arteriole (smaller arrow) Dark area at top right corresponds to the glomerular tuft of these arterioles × 350

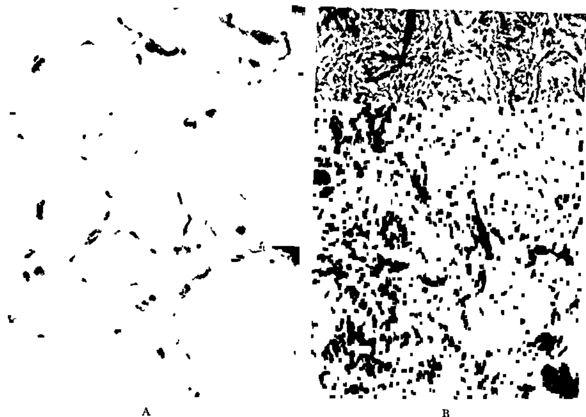


Fig 7 A Fluorescence microphotograph from the stenosed kidney of a rat hypertensive throughout 3 months (Group III C) There is an abundant network of fluorescent varicose fibres as the result of a relative predominance of vessels in the contracted parenchyma In bottom right corner a glomerular tuft is present × 300

Fig 7 B Neighbouring histological section of the area depicted in Fig 7 A showing the fibrous contraction of the parenchyma which contains meandering arteries and arterioles and a number of glomeruli in various stages of degeneration No tubules are seen van Gieson elastin × 200

followed by a re-innervation It should be recalled, however, that the interval between the production of the stenosis and the development of hypertension usually varies considerably and this feature was recorded in the present investigation Thus, at the time of killing the rats of Group III A had been alive with their stenosis for periods ranging from 8 up to 34 days, and those of Group III B for periods ranging between 13 and 38 days It seems unlikely that any denervation and re innervation effect would become evident at such highly different intervals following the operation Moreover, a significant number of stenosed kidneys from normotensive rats (Group II) did not show any innervation alterations, although the stenosis had been efficient enough to produce parenchymal alterations

On the basis of the above considerations it may be assumed that the decrease in fluorescence intensity and number of fluorescent nerve terminals reflects a depletion of the transmitter content of the nerve terminals of the undenervated organ Such a depletion of the transmitter has been observed in the spleen during haemorrhagic shock (*Dahlström & Zetterström* 1965) and in the heart and kidney of mice with experimental ascites (*Tallqvist et al* 1970) In the latter event the angiotensin-aldosterone system was suggested to be involved This system may well

have contributed to a depletion of the transmitter in the early hypertensive rats of the present material since angiotensin potentiates the release of noradrenalin during sympathetic nerve stimulation (*Schumann et al* 1970), the plasma renin and hence plasma angiotensin is increased during the early phase of renovascular hypertension (*Koletsky* 1967) and data suggesting an increased activity of the sympathetic innervation of peripheral vessels in rats with renal hypertension have been presented (*Graham et al* 1970) In agreement with our findings the most marked release of the transmitter will then occur at the juxtaglomerular level of the stenosed kidney where the angiotensin level is particularly high This transmitter release may further contribute to the maintenance of high plasma angiotensin levels by directly stimulating the renin producing juxtaglomerular cells

Whether the initial rise in plasma renin and plasma angiotensin levels is brought about by nervous or extranervous stimulation of the juxtaglomerular cells or by a combination of stimuli is unknown (*Tobian* 1966) and cannot be assessed on the basis of the present findings Some elucidation of this question may however be gained from an analysis of the findings in the operated rats which remained normotensive (Group II) In order to cover the range of the period from operation to killing of the hypertensive rats some rats of group II had to be killed long before the possible latent period for the development of hypertension had elapsed and three of these rats (killed 10 14 and 30 days after operation) showed nerve alterations in their stenosed kidney similar to those observed in the hypertensive rats This suggests that the nerve alterations may precede the development of hypertension and therefore possibly be of pathogenetic significance The findings related to the three normotensive rats agree with the report by *de Champlain et al* (1969) of a reduced norepinephrine storage in the heart and kidney during the pre hypertensive phase of rats rendered hypertensive by DOCA saline administration The absence of adrenergic nerve structures in the stenosed kidneys of five operated normotensive rats is irrelevant since these kidneys displayed complete infarction

The normalization of the sympathetic nervous pattern in the stenosed kidney after more than one week of hypertension is in agreement with the common observation that the plasma renin activity, and hence the plasma angiotensin level, tends to reassume normal levels when hypertension is prolonged (*Koletsky et al* 1967) This has been interpreted as indicating that extrarenal factors will take over and keep the blood pressures high once it has become elevated through renal mechanisms (*Page* 1968)

The abundance of intensely fluorescent varicose fibres which was a regular feature of the stenosed kidney from rats with prolonged hypertension (Group III C) may either represent an over loading of the adrenergic nerve terminals by transmitter substance or be a non specific feature secondary to the contraction of the parenchyma with consequent crowding of nerve structures The present study would not form a basis for a solution of this problem which seems to deserve further investigations

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This study was supported by the *Swedish Medical Research Council* (Project No B69–12X 716 04A) and the *Swedish National Association against Heart and Chest Diseases*

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Acta path microbiol scand Section A *80*, 47–53 1972

CYTOLOGY BY THE FILTER IMPRINT TECHNIQUE

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A modification of Volet s filter imprint technique was applied to a number of cytological specimens The imprint technique was compared with the original membrane filter technique with a view to cellular density, cell preservation and detail, as well as diagnostic accuracy The result shows that the imprint method is quick, simple to perform, and in respect to diagnostic accuracy it is equal to or even superior to the standard membrane filter method Lastly, as far as urinary specimens are concerned, both methods appear to give somewhat more accurate diagnostic results than the conventional centrifugation smearing technique, although this aspect was not thoroughly investigated

To obtain an increased diagnostic accuracy in cytological studies attempts have been made to supplement the conventional sedimentation technique using centrifugation by a modification of the membrane filter technique This has been applied to serous, aqueous samples (urine, washings, aspirate from the prostate), proteinous fluids (pleural fluid, ascites), and mucinous secretions (bronchial secretion, secretions from the oesophagus, stomach, and duodenum) (*Fischer* 1970)

This has been of quite particular importance in urinary cytology which makes up a large part of the work done in cytology

In many places abroad, the membrane filter technique is used as the only method in urinary cytology, as several comparative studies have demonstrated the superiority of this method above sedimentation after centrifugation and smearing, both with respect to quality and diagnostic accuracy (*Kern et al* 1966)

In this country, the membrane filter method has not gained much ground although applicable results have been reported (*Iversen et al* 1968)

The main reasons why its results have not been up to the expectations are

(1) That often only small quantities of the sample can be sucked through the filter before it gets occluded
(2) That often it is difficult to dissolve the filter entirely, and therefore the contrast between cells and background is reduced (*Iversen et al* 1968)
(3) That the method is fairly time consuming, because the process has to be watched

Our attempt at modifying the membrane filter technique with a view to eliminating these disadvantages was based upon the so called filter imprint technique (*Volet* 1965)

Received 4 x 71

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MATERIALS AND METHODS

Standard membrane filter preparations and filter imprint preparations from a number of cytological media, mainly urine and washings, were compared

Most interest was focussed on the following three characteristics

(1) Cellular density
(2) Cell preservation and detail
(3) The diagnostic grading of the cells by accepted definitions (*Bergkvist et al* 1965)

The material comprised all specimens received for cytological study during a period of 6 weeks

90 samples of urine from 45 patients
14 samples of gastric juice from 8 patients
6 samples of duodenal juice from 2 patients
2 samples of bronchial secretion from 2 patients
2 samples of pleural effusion from 2 patients

To all samples of freshly voided urine, cytological fixative, in the form of an aqueous solution of methanol acetic acid (*Tjernberg et al* 1965), was immediately added, at the ratio 2 parts urine to 1 part fixative Other media were treated without primary fixation

After shaking each sample was divided into three portions

(1) 10 ml for the standard membrane filter (SMF) technique
(2) 10 ml for the membrane imprint (MI) technique
(3) The remainder for ordinary centrifugation and smearing of sediment (C)

Re (1) Standardized Membrane Filter Technique

10 ml fluid was sucked through a rectangular filter, pore size 8 μ (vacuum 5–10 mm of mercury)

The filter was fixed in 70 per cent alcohol washed in water, and stained for 5 minutes in Mayer haematoxylin washed in water, and then stained with eosin for 10 minutes Washed and dehydrated in alcohol

The filter was mounted on an egg white coated slide, the cell side next to the glass

The filter was dissolved by ethyl acetate in the course of about one hour and dried Then xylol was added and a cover slip applied

Re (2) Filter Imprint Technique

10 ml was centrifuged for 5 minutes at 1000 r p m The supernatant was decanted and replaced by the same quantity of 70 per cent alcohol The sample was shaken several times to dissolve the sediment

Thereafter, the sample was sucked through a rectangular filter, pore size 8 μ using light vacuum (5–10 mm of mercury)

At the very moment that the last portion of the sample had passed the filter the upper part and funnel of the apparatus were quickly removed as

TABLE 1 *Cellular Density*

Cellular density	4	3	2	1	0	Total
Urine						
SMF	30	28	21	7	4	90
MI	34	31	19	2	4	90
Gastric juice						
SMF	6	3	3	2	0	14
MI	8	2	4	0	0	14
Duodenal juice						
SMF	2	2	2	0	0	6
MI	2	1	2	1	0	6
Bronchial secretion						
SMF	1	1	0	0	0	2
MI	2	0	0	0	0	2
Pleural effusion						
SMF	1	0	1	0	0	2
MI	0	1	1	0	0	2

SMF – standard membrane filter
MI = membrane imprint
Code of density
0 = no cells present
1 = 0 1 cell in the field 1 400
2 – 2 5 cells in the field 1 400
3 – 5 20 cells in the field 1 400
4 – more than 20 cells in the field 1 400

TABLE 2 *Cell Preservation*

Preservation	3	2	1	0	Total
Urine					
SMF	12	53	17	8	90
MI	44	37	9	0	90
Gastric juice					
SMF	3	8	3	0	14
MI	3	9	2	0	14
Duodenal juice					
SMF	0	3	3	0	6
MI	0	5	1	0	6
Bronchial secretion					
SMF	0	2	0	0	2
MI	0	2	0	0	2
Pleural effusion					
SMF	0	2	0	0	2
MI	0	2	0	0	2

SMF = standard membrane filter
MI = membrane imprint
Code of cell preservation
0 = too poorly preserved for evaluation
1 = poor preservation and detail
2 = fair preservation and detail
3 = good preservation and detail

TABLE 3 *Diagnostic Assessment, Including Grading of Tumour Cells in the Urine*

	Neg	Pos	Grade I	Grade II	Grade III	Grade IV	Susp
Urine							
SMF	65		6	11	5	3	0
MI	65		6	9	7	3	0

	Neg	Pos	Susp
Gastric juice			
SMF	12	0	2
MI	12	0	2
Duodenal juice			
SMF	6	0	0
MI	6	0	0
Bronchial secretion			
SMF	1	1	0
MI	1	1	0
Pleural effusion			
SMF	2	0	0
MI	2	0	0

drying of the filter has to be avoided Possibly it may be constantly moistened with 70 per cent alcohol

A cold, egg white-coated slide was quickly applied, its coated surface next to the filter

When the slide is lifted, the filter adheres The slide was turned and pressed against dry filter paper The filter was then removed with a pincette and the cellular layer now adhering to the slide was treated as follows

Fixed in ether alcohol for 5 min
Washed in running water for 5 min.

Stained in Mayer haematoxylin for 10 min
Washed in running water for 5 min
Stained with eosin for 5 min
Washed in water
Dehydrated with alcohol
Xylol was added and a cover slip applied

RESULTS

The results are given below in Tables 1, 2, and 3

DISCUSSION

(1) *Cellular Density*

As is apparent from the tabulations the cellular density in the imprint preparations was equal to that found with the membrane filter technique, regardless of the nature of the medium

The finding that the cell count might even be higher in the imprint than in the corresponding membrane filter preparations must indicate that the filter often becomes occluded in the current membrane filter procedure before the entire sample (10 ml) has been sucked through In the imprint method, on the other hand, the initial centrifugation followed by suspension in 70 per cent alcohol practically always secures suction of the entire sample, so that the total number of cells will be trapped

In the great majority of cases only a few no cells were left on the filter after the imprint had been made As far as the urine concerned, 63 per cent of the filters contained less than 10 per cent of the total number of cells on the imprint With respect to the gastric and duodenal specimens, the yield was somewhat lower, 50 per cent and 33 per cent, possibly due to the higher viscosity of these samples, resulting in greater adhesion between the filter and the cells

The number of bronchial and pleural secretions was too small to permit any final assessment

(2) *Cell Preservation and Detail*

In this respect there was a distinct difference between the usual membrane filter technique and the imprint technique This was most evident in the urinary specimens, where 48 per cent of the imprints were found to be of group 3, *i e* good or excellent preservation and detail, while this applied to only 13 per cent of the membrane filter specimens For the other media the difference was not quite so striking

The better quality of the imprint preparations was due primarily to greater contrast between the background and the cells As already mentioned, it may often be difficult to dissolve the protein imbibed filter It has tendency to retain dye which mordants th filter and protects it from being dissolved

In the standard membrane filter techniqu the inflammatory and epithelial cells ar often seen on a thick background of cellula debris, deposited protein, remnants of re blood cells, and in the case of urine als precipitations of urate

This background is considerably reduce in the imprint technique, no doubt becaus the named components are left in the pore of the filter, while only the purely cellula elements are transferred by the imprint

Fig 1 shows cytologic details employing the various techniques

(3) *Diagnostic Assessment, Including Grading of Tumour Cells in the Urine*

The diagnostic results were largely coincident However, the cells in two urinary specimens were assessed as grade III tumour cells in the imprint preparations while, by the usual membrane filter method, the cells from the same specimens had been declared to be grade II tumour cells Biopsy from the bladder, removed immediately after the urinary samples had been voided, showed grade

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Fig 1
(a) Membrane imprint method Fragment of grade I papilloma from urine 250 1
(b) Membrane imprint method Grade II tumour cells from urine 250 1
(c) Membrane imprint method Grade III tumour cells from urine 250 1
(d) Membrane imprint method Grade III tumour cells from urine 250 1

Stained in Mayer haematoxylin for 10 min
Washed in running water for 5 min
Stained with eosin for 5 min
Washed in water
Dehydrated with alcohol
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III transitional cell carcinoma in both cases

In this country, *Fischer* (1970) has recently demonstrated that the membrane filter method is a valuable supplement to the conventional centrifugation technique and at times superior to the latter in diagnostic accuracy, especially in urinary cytology

As will appear from the above, the imprint method affords at least as good diagnostic results as the standard membrane filter technique to which it is superior owing to a much more distinct cell structure and a pure, clear background

However, its main advantage is that it is very simple and quick With a trained staff it takes only 8–10 minutes to make the specimen ready for fixation and staining, as the slow dissolving of the filter in the other membrane filter methods is avoided This has proved of great practical value in a Dept of Pathology where cytology is making up an ever increasing part of the work

Concurrently with the investigation the remainder of the samples was used for conventional centrifugation and smearing of sediment on 4 slides

It would have been desirable to make direct comparison of these smears (C) with standard membrane filter (SMF) and membrane imprint (MI) preparations with respect to cellular density, cell preservation and detail as well as diagnostic assessment However this was done only as far as the cytological diagnosis was concerned

The volume of the residual samples ranged from 20 to 170 ml, so that the cellular density in the centrifuged smears could not be compared directly with that in the standard membrane filter and imprint preparations

Fig 1

(e) Standard membrane filter method Grade II tumour cells from urine Note the packed, crackled background 100 1

(f) Standard membrane filter method Grade III tumour cells from urine 100 1

(g) Standard membrane filter method Grade III tumour cells from urine 250 1

(h) Centrifugation smear preparation Grade III tumour cells from urine 250 1

Unlike the haematoxylin eosin staining of the standard and imprint preparations, the centrifuged smears were routinely Papanicolaou stained Therefore, no comparison was made with respect to cell preservation and detail

As for diagnostic assessment, there was only a certain discordance as far as the urinary specimens were concerned

Centrifuged smears from 4 samples of urine were assessed as negative, while the corresponding standard membrane and membrane imprint preparations showed grade I tumour cells A subsequent biopsy was available from only one of these cases It showed grade I papilloma of the bladder

Furthermore centrifuged smears from 4 samples of urine were assessed as suspicious of tumour cells while the corresponding standard membrane and membrane filter preparations showed grade II tumour cells In two of the cases, biopsy specimens were examined later, both showed grade II papilloma of the bladder

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ULTRASTRUCTURE OF THE ALVEOLAR WALL IN EXPERIMENTAL PARAQUAT POISONING

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Electron microscopic studies of the rat lung were carried out at various intervals after subcutaneous injection of paraquat Within 12–18 hours after paraquat injection, capillary engorgement and interstitial oedema were noted in alveolar walls After 24 hours there was, in addition, mitochondrial degeneration and cytoplasmatic vacuolization in granular pneumo cytes, membraneous pneumocytes, and capillary endothelial cells Desquamation of necrotic alveolar epithelial cells was a characteristic feature 48 hours after injection of paraquat Many of these desquamated cells were identified as granular pneumocytes from their preserved lamellar inclusions The severe ultrastructural lesions of the alveolar epithelium may serve to explain the derangement of pulmonary surfactant which has been observed in paraquat poisoning under similar experimental conditions

The pulmonary lesions which can be induced in animals, including man, by poisoning with the herbicide paraquat (1 1′ dimethyl-4 4′ bipyridylium dichloride, Gramoxone, ICI) consist of atelectasis, intra-alveolar oedema and haemorrhage, hyaline membranes, fibrosing interstitial pneumonia, and proliferation of bronchiolar epithelium (2, 5 7, 12, 14, 18, 19) Areas with collapsed alveoli characteristically display overdistension of bronchioles and alveolar ducts, which causes a microscopic appearance similar to "atelectasis of prematurity" (cf 11) As pointed out by *Manktelow* (17), this feature suggests that paraquat-induced alveolar collapse is secondary to deficiency of surface active phospholipids ("surfactant") in the alveolar lining layer Recent studies of the surface properties of alveolar wash in experimental paraquat poisoning have confirmed this hypothesis, in showing that functional derangement of pulmonary surfactant precedes the development of atelectasis (21, 22)

The synthesis of pulmonary surfactant is generally attributed to the granular pneumocytes (4, 13) The present study was undertaken in order to investigate whether the functional derangement of pulmonary surfactant in paraquat poisoning is related to ultrastructural changes in the granular pneumocytes or other components of the alveolar wall Preliminary results have been reported (20)

Received 3 vi 71

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MATERIAL AND METHODS

The experimental series consisted of five female Sprague Dawley rats (appr 200 g) which were given a subcutaneous injection of paraquat, 35



Fig 1 Ultrastrn cture of alveolar wall 18 hours after injection of paraquat There is prom nent capil lary engorgement but the granular pneumocytes (*top right*) appear normal Interstitial oedema though absent in this field was in other areas consp cuous comparable to that shown in Fig 2 × 10 000

mg/kg body we ght The animals were killed with intraperitoneal Nembutal at 4 12 18 24 and 48 hours after the administrat on of paraquat Un treated rats of correspond ng s ze sacrificed with intraperitoneal Nembutal served as controls

In order to prevent postmortem alveolar collapse the lungs were f xed *in s tu* by tracheal instillation of 10 cc cold (4°C) 2 per cent glutaraldehyde cacody late buffer (pH 7 4) Thereafter the trachea was tied and the thorax was opened The lungs were quickly but carefully removed and immersed in 2 per cent glutaraldehyde buffered as above After fixation for 3 hours at 4°C mult ple small tissue blocks measuring about 1×1×1 mm, were cut from both lungs rinsed in cacodylate buffer (pH 7 4) for 3 hours and osm cated in veronal buffered (pH 7 4) 1 per cent osm um tetroxide at 4°C for 1 hour The tissue blocks were then rinsed in veronal buffer (pH 7 4) for 30 m nutes dehydrated for 1 hour in acetone and embedded in Vestopal W Sections were cut with glass knives on an LKB III Ultrotome and examined with a S emens I A electron m croscope In all sections a combined lead citrate and uranyl acetate stain ng was ap plied

RESULTS

The fixation and staining methods used proved to be adequate as regards the epithe lium the interstitial tissue and the capillaries of the alveolar walls These structures were well preserved in the normal animals How ever the alveolar lining layer was not visualized

In the paraquat treated animals, ultra structural lesions were present after 12 but not after 4 hours These early changes con sisted of irregular interstitial oedema and engorgement of alveolar capillaries The same features characterized the alveolar walls of the animal sacrificed 18 hours after the ad ministration of paraquat (Fig 1)

Twenty four hours after the injection of paraquat, interstitial oedema (Fig 2) and ca pillary engorgement were more prominent al though still patchy, and there were also

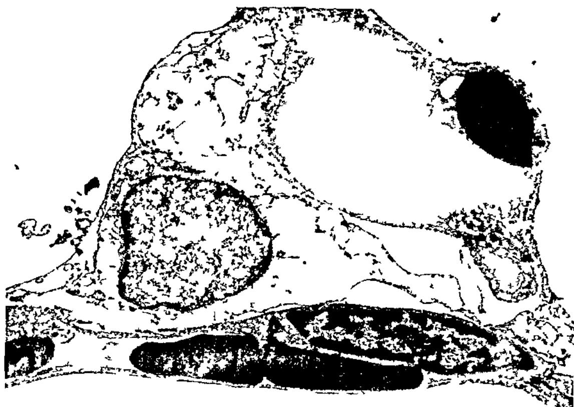


Fig 2 Pronounced interstit al oedema in alveolar wall 24 hours after injection of paraquat Cap llary engorgement a prominent feature in other parts of the same lung is not impress ve in th s f eld × 15 600

lesions of the alveolar epithelium and the capillary endothelium Thus the cytoplasm of granular pneumocytes membraneous pneumocytes and capillary endothelial cells owed vacuolization and the mitochondria of these cells were swollen and apparently degenerating It should be pointed out that these mitochondrial alterations were not an artifact caused by inadequate fixation since similar changes were absent in the controls In the experimental rats lamellar inclusion bodies were well preserved in otherwise degenerating granular pneumocytes (Fig 3)

In the animal sacrified 48 hours after the injection of paraquat the epithelial and endothelial lesions (Fig 4) were more pronounced with widespread necrosis of the alveolar epithelium affecting granular as well as membraneous pneumocytes Desquamated alveolar epithelial cells with advanced degenerative features were frequently found in the alveolar spaces Many of these ghost cells contained easily recognizable lamellar inclusions and could hence be identified as necrotic granular pneumocytes (Fig 5)

COMMENT

The bipyridylium compounds can accept electrons from any system with appropriate redox potential and then undergo reoxidation at the expence of molecular oxygen which is reduced to hydrogen peroxide (24) Well oxygenated cells as those comprising the alveolar wall are probably particularly sus ceptible to this type of biochemical disturbance This might account for the fairly selective toxic effects of paraquat on the pulmonary parenchyma

It is obvious from the present study that the early alveolar lesions in paraquat poisoning are not confined to the epithelial cells

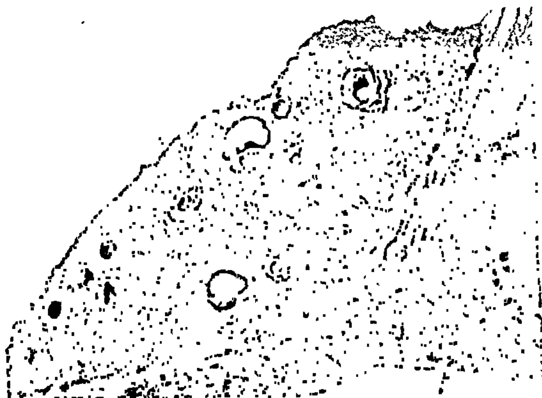


Fig 3 Degenerating granular pneumocyte 24 hours after injection of paraquat The mitochondria are swollen, and their laminar structure is disrupted The osmiophilic lamellar inclusions, however, are well preserved × 22,500

but include the interstitial tissue and the capillary endothelial cells as well Similar findings were recently reported by *Vijeyaratnam* & *Corrin* (26), although these authors concluded that endothelial cell damage is not a characteristic feature of the disease Our results also tally well with recent electron microscopic observations by *Kimbrough* & *Gaines* (14), who reported advanced degeneration of alveolar lining cells 48 hours after oral administration of comparatively high doses of paraquat (400 mg/kg body weight) Some cytoplasmatic swelling of membraneous pneumocytes was noted by these authors as early as 4 hours after the administration of paraquat

The degeneration of alveolar epithelium, which we found manifest 24 hours after the injection of paraquat (35 mg/kg body weight), serves to explain the derangement of pulmonary surfactant which is a regular feature of paraquat poisoning under similar experimental conditions (22) However, reduced surface activity in alveolar wash can be demonstrated as early as 2–4 hours after the administration of paraquat (21), i e at a stage when the ultrastructural appearance of the alveolar wall, according to our present findings, is still normal This very early effect on the surfactant system of the lung is possibly caused by a direct influence of paraquat on the phospholipid metabolism of the granular pneumocytes By peroxidation of fatty acids (8), paraquat might interfere with the synthesis of dipalmitoyl lecithin a major constituent of pulmonary surfactant Such a noxious effect should be reflected in the surface properties of alveolar wash within considerably less that 24 hours, which is the estimated turnover rate of pulmonary lecithin in the rat (25)

The lamellar inclusions of the granular



Fig 4 Severe endothelial lesion in alveolar capillary 48 hours after injection of paraquat The endothelial cell indicated by *arrow* displays pronounced cytoplasmatic swelling Leucocyte in capillary lumen (*lower right*) × 27 000

pneumocytes are generally believed to represent accumulation of lipids which are to be extruded in the alveolar lining layer as part of the pulmonary surfactant system (13 15 23) The preservation of these inclusions in otherwise degenerating granular pneumocytes would therefore seem to contradict speculations concerning the specific effect of paraquat on the surfactant metabolism of the lung However the osmophilic component of these lamellar inclusions probably represents unsaturated phospholipids rather than dipalmitoyl lecithin (1) In order to visualize the latter compound other fixation methods such as tricomplex flocculation (9 10) have to be applied The lamellar inclusions which in the present study were visualized in degenerating granular pneumocytes with osmium fixation may thus represent inactive precursors of pulmonary surfactant

In a few specimens not included in the present series ruthenium red (1 mg/ml) was added to the fixation fluid (3 16) This modification of the technique visualized the glycocalyx of the alveolar epithelium and our preliminary observations (20) seemed to indicate that this mucopolysaccharide coating of the alveolar surface was reduced in paraquat treated rats However our initial findings were not consistent in subsequent experimental series Further modifications of the method will obviously be required in ultrastructural studies of the alveolar lining layer in paraquat poisoning These modifications include perfusion or immersion fixation under positive endotracheal pressure to preserve the air liquid interphase of the alveolar surface (cf 27) and tricomplex flocculation for fixation of saturated phospholipids such as dipalmitoyl lecithin

As originally suggested by *Manktelow* (17) paraquat poisoning might be adopted as an

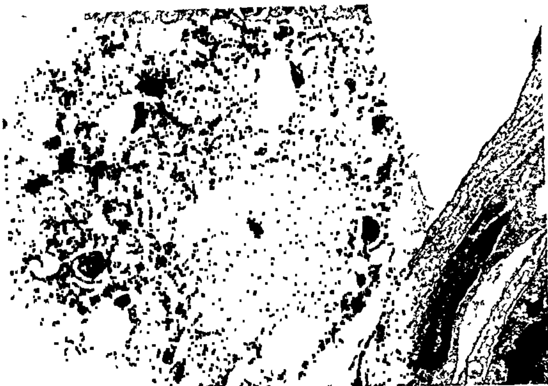


Fig 5 Desquamated necrotic cell in alveolar space 48 hours after injection of paraquat This "ghost cell" is identified as a granular pneumocyte from its well preserved lamellar inclusions × 19,000

experimental model of the idiopathic respiratory distress syndrome In particular, the early stages of the paraquat-induced pulmonary lesions provide useful material for studies of the relation between surfactant deficiency and alveolar collapse (21, 22) At a later stage however, the picture is obscured by inflammatory changes and reparative features Furthermore the analysis of surface properties of alveolar wash is rendered less accurate after the initial phase of the disease, since surface active molecules to some extent probably become adsorbed to the desquamated epithelial cells (6) This might give a false impression of low surfactant content, if the wash is analysed with physical methods, such as pulsating bubble or Wilhelmy balance

This work was supported by grants from *The Swedish Medical Research Council* (Project No K71-12X 3351 01), *The Swedish National Association against Heart and Chest Diseases* and *"Karolinska Institutets Reservationsanslag för Ograduerade Forskare"*
Crystalline paraquat was generously provided by ICI Industrial Hygiene Research Laboratories, Alderley Park, Nr Macclesfield, Cheshire, England

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TABLE 2 *Chromosome Counts in the Neurinomas*

Neurinoma no	Chromosome numbers 41	42	43	44	45	46	47	48	53	88	89	92	Total cells	
N 1	–	1	–	1	48	–	–	–	–	–	–	–	50	
N 2	1	2	–	3	15	25	1	–	–	1	–	2	50	
N 3	1	–	–	–	10	34	2	–	1	–	1	1	50	
N 4		–	1	1	3	26	5	2	–	–	–	–	38	9
N 5	–	–	–	2	2	43	3	–	–	–	–	–	50	10
N 6		–	–	–	1	54	–	–	–	–	–	1	56	7
N 7		–		–	–	50	–	–	–	–	–	–	50	7

showeds a loss of 1 G chromosome (Fig 1 a) In addition, one of the 45 chromosome cells showed a loss of 1 E17–18 and a gain of 1 t marker This acrocentric marker was a little smaller than the smallest D chromosomes It seemed probably that it was derived from the lost E chromosome The cell with 2x=44 differed from the S cells by the loss of 1 C chromosome The other variant cell, with 2x=42 differed from the S cells by the loss of 1 C chromosome and 2 chromosomes E17–18

N 2 – This spinal neurinoma had an S=46 The spread in the modal region ranged from S–5 to S+1 Three tetraploid cells had chromosome numbers in accordance with doubling products of elements in the S region Eleven 46 chromosome cells were karyotyped Ten of these had the same karyotype characterized by the loss of 1 E17–18 and the gain of 1 t marker (Fig 1 b) The acrocentric marker had a size intermedia between that of the smallest D and the big gest G chromosomes, the short arm did not seem to be satellited It was reasonable to assume that the marker was derived from the lost E chromosomes

The deviating 46 chromosome cell differed from the S cells by the loss of 1 C chromosome and the gain of 1 small m marker (arm index about 1 1) The size of the metacentric marker was similar to that of the G chromo somes A similar m marker was also found in the single cell with 2x=47, its karyotype was otherwise the same as that of the S-cells All of the 7 karyotyped cells with 45 chrom somes differed from the S by the loss of 1 C chromosome (Fig 1 c), these elements con stituted a sideline One variant cell with 2x=44 was analysed, it differed from the s cells by the loss of 1 G chromosome The other analysable variant cell, with 2x=42,

TABLE 3 *Karyotypic Features in the Neurinomas*

Neurinoma no	S or s number	Karyotype: Ordinary chromosome groups	Markers	Frequency of S– or s–cells per cent
N 1	S=45	1G	–	91 4
N 2	S–46	1E17 18	+ 1t	45 5
	s=45	1C 1E17–18	+ 1t	30 0
N 3	S=46	–	–	68 0
	s 45	1C	–	17 1
N 4	S 46	–	–	68 4
N 5	S=46	1C –1E17–18 –1G	+ 1st$_1$ + 1st$_2$ + 1sm	71 7
N 6	S=46	1B –1C	+ 1st$_1$ + 1m	96 4
N 7	S=46			100 0

differed from the s by the loss of 1 G and 2 D chromosomes The polyploid elements could not be karyotyped

N 3 – This was the second neurinoma with a spinal location, and it also had a 46-chromosome stemline As in the previous case, the spread was mainly hypomodal and it ranged from S–5 to S+7 A few doubling products of modal elements occurred in the tetraploid region

Thirteen cells with the *S* number were karyotyped, all of these had the normal, diploid complement Seven cells with 45 chromosomes were analysed, one of these showed a loss of 1 D chromosome but all the others differed from the normal by the loss of 1 C chromosome (Fig 1 d) The latter cell group formed an s The 2 cells with 2x=47 both showed a gain of 1 C chromosome The elements with 2x=42 and 2x=53 could not be karyotyped The single analysable tetraploid cell, with 92 chromosomes, had a karyotype in accordance with a doubled S cell

N 4 – This brachial neurinoma had its mode at S=46, and the spread ranged from S–3 to S+2 All of the 12 analysed cells with 46 chromosomes had the normal, diploid karyotype Four hypomodal cells were also karyotyped and they showed losses in groups C and/or D

Three of the 47 chromosome cells could be karyotyped, one of these showed a gain of 1 t marker and the other two had gained 1 small m marker (arm index about 1 2) The acrocentric marker was approximately half as big as the smallest G chromosome, in both cells the metacentric marker had a size similar to that of the smallest G chromosome (Fig 1 e) The two 48 chromosome cells differed from the normal by the gain of 2 similar t markers having the same size as that in the cell with 2x=47 The origin of the t and m markers was obscure

N 5 – This acoustic neurinoma had a prominent mode at S=46, and the restricted spread ranged from S–2 to S+1 Eighteen cells with the S number were analysed and 15 of these had the same karyotype This was characterized by the loss of 1 C, 1 E17–18 and 1 G chromosome and the addition of 3 different markers, st_1, st_2 and sm, respectively (Fig 1 f) The st_1 marker (arm index about 4 3) had a size intermediate between that of the biggest G and the smallest D chromosomes The st marker (arm index about 4 0) had a size similar to that of the biggest D chromosomes The sm marker (arm index about 3 0) was about twice as big as the biggest D chromosomes

One of the deviating 46-chromosome cells differed from the S cells in that it had a normal number of C chromosomes and showed a loss of 2 G chromosomes The other 2 variant cells, with 2x=46, had no markers but an abnormal representation in groups C, G and A3, C, E17–18, G, respectively The 4 cells with hypomodal numbers, as the 3 cells with 2x=47, were closely related to the S cells, the differencies being caused by a deviating representation in the groups C, G and/or a loss of the sm marker

The exact origin of the 3 markers was somewhat uncertain It seemed reasonable, however, to assume that the lost C, E and G chromosomes had participated in the formation of the markers There were no signs of further structural rearrangements Thus, the markers were morphologically stable components of the S karyotype, and the frequency of breakage was low, about 7 per cent, as in almost all of the other neurinomas (N 7 was an exception, see below)

N 6 – The second acoustic neurinoma also had a 46 chromosome stemline Only 2 cells had other chromosome numbers viz 45 and 92 Fourteen cells with the S number were karyotyped They all differed from the normal in the loss of 1 B and 1 C chromosome and the gain of 1 st marker and 1 m marker (arm index about 4 2 and 1 2, respectively) (Fig 1 g) Morphologically the st marker resembled a B chromosome but it was approximately 25 per cent longer than the longest members of this group The m marker had a size similar to that of the chromosomes E16 but its centromere had a more median location than the members of pair E16 A

translocation between one B and one C chromosome was a plausible mechanism for the origin of the 2 markers

The single hypomodal cell differed from the S by the loss of 1 *further C chromosome* The tetraploid cell had a karyotype in agreement with that of doubled S cell

N 7 – This was the third spinal neurinoma, and it occurred in a patient with von Recklinghausen's disease All cells counted had 46 chromosomes Fifteen of them were karyotyped, and they all *showed the normal*, diploid complement

Breaks were unusually common in this tumour Approximately three quarters of the counted cells showed one or more breaks These were usually of the chromatid type (Fig 1 h) No particular chromosome type was affected more often *than the others* The reason for the high frequency of breakage in one particular neurinoma was obscure

II *The Neurosarcoma*

Table 4 shows the chromosome counts in the fixations from the neurosarcoma The findings in D and Pc agreed, and they showed a flat mode at 79–80, close to the border between hypertriploidy and hypotetraploidy *Almost all counts* were found within these two regions but the spread was considerably d in comparison with the neurinomas

Altogether 31 cells were karyotyped from the two fixations, and 27 of them belonged to the modal region None of these modal elements had the same karyotype. This extraordinary pleomorphism was *mainly* due to extensive structural rearrangements As far as the ordinary chromosome groups were concerned, however there were only minor differences between most analysed cells In this respect the findings were as follows (in relation to a doubled normal, female cell) +1A3, +1–2B, –10–14C, –6–7D, –1E16, –3–5E17–18, –3–4F, –2–3G

The number of markers in the modal cells ranged from 7 up to as many as 20, and most cells had 12–14 markers Disregarding the variety of morphological marker types observed in one or a few cells, the following types were encountered in most karyotyped cells Im (arm index about 1 2, the size about 40 per cent of the average size of the A1 chromosomes), 2st, (arm index about 4.5, the size about 90 per cent of A1), 1–2st (arm index about 5 2, the size about 70 per cent of A1), 1t (the size about 30 per cent of A1), 1–2 ring chromosomes (a more constant, small one having a size similar to that of the arms of the A1 chromosomes, and a less common, big ring chromosome, somewhat bigger than the A1), 1 dicentric marker (inconsistent morphology and usually bigger than the A1) and 3–8 minute markers The minutes were of m-, sm-, st and t type but due to their *small size* (*ranging from about* two thirds of a G chromosome to the border of visibility) it was only possible to observe that all these morphological types were represented in the group The karyotype of two cells with the modal number, 79, and one hypermodal cell, 2x=85 are illustrated in Fig 2

The single cell with 154 chromosomes could not be analysed in detail The occurrence of similar marker types as in the modal *cells, however, indicated that it was derived* from a doubled cell in this region All of the cells with 2x=46 could be analysed They had the normal, diploid complement and

TABLE 4 *Chromosome Counts in the Neurosarcoma*

Method of fixation	Chromosome numbers 46	65	73	74	75	76	77	78	79	80	81	82	83	84	85	154	Total cells
D	1	1		2	1	2	3	3	12	10	7	6	–	1	1		50
Pc–7	3		1	2	3	2	5	5	15	11	5	4	3	1	1	1	62
Total	4	1	1	4	4	4	8	8	27	21	12	10	3	2	2	1	112

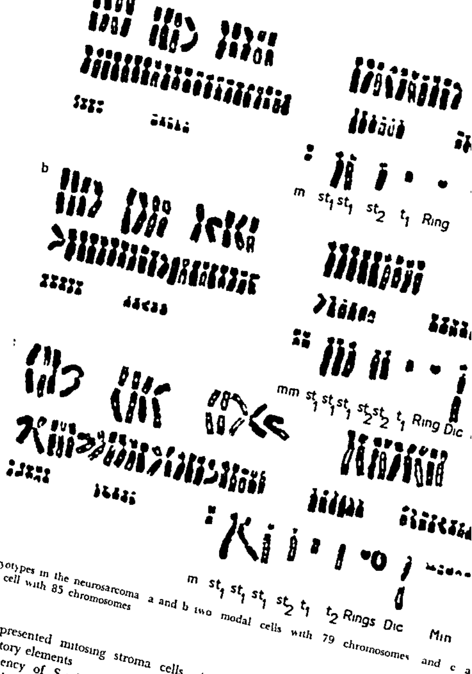


Fig 2 Karyotypes in the neurosarcoma a and b two modal cells with 79 chromosomes and c a hypermodal cell with 85 chromosomes

probably represented mitosing stroma cells or inflammatory elements

The frequency of S cells in the neurosarcoma could not be determined because of the absence of a definitely predominating karyotype among the analysed modal cells. The mode at 79–80 corresponded to a group of closely related cells which all differed slightly from each other and which seemed to possess a similar competetive capacity. This extreme picture was not observed in the previous study of 53 malignant gliomas in adults (*Mark* 1971 c). The gliomas with triploid and tetraploid stemlines, however, showed a very low frequency of S cells, in some cases the frequency was even below 10 per cent, and the picture was reminiscent of that observed in the present sarcoma

TABLE 5 *Mean Differences between Observed and Expected Values*

Material	Number of tumours	Chromosome types										Markers
			A		B	C	D	E		F	G	
		1	2	3	4–5	6–X–12	13–15	16	17–18	19–20	Y–21–22	
Neurinomas												
all tumours	7	+0 0	+0 0	+0 0	—0 1	—0 2	+0 0	+0 0	—0 3	+0 0	—0 3	+ 0 9
tumours with markers	3	0	0	0	—0 3	—0 7	0	0	—0 7	0	—0 3	+ 2 0
The neuro sarcoma	1	+0 6	+0 6	+1 7	+1 1	—6 9	—5 1	—0 7	—2 1	—2 0	—0 9	+13 7
Meningomas (Mark, 1970 a)	21	—0 0	—0 0	+0 1	+0 1	+0 1	—0 2	+0 0	—0 0	+0 2	—0 9	+ 0 8
Pituitary adenomas (Mark, 1971 b)	7	—0 2	—0 4	+0 2	+0 0	+0 5	—0 2	—0 0	—0 2	+0 4	—0 2	+ 0 1

DISCUSSION

In the present series of neurinomas there was a diploid hypodiploid stemline pattern The tendency towards a hypodiploid evolution in these neoplasms was also indicated by the predominance of hypodiploid variant cells, and, above all, by the occurrence of both the observed sidelines (in N 2 and N 3) in this region The S pattern in the neurinomas was different from that previously found in the pituitary adenomas (*Mark* 1971 b), but it resembled the S distribution observed in the meningomas (*Mark* 1970) The latter tumour type, however, showed a more extensive hypo diploid variation, and, in addition, the stem line pattern was characterized by a peak at S=45

Karyologically normal, diploid stemlines and pseudodiploid stemlines predominated in the neurinomas, each category being found in 3 tumours In ordinary chromosome groups, the deviations (always losses) were confined to the groups B, C, E17–18 and G A similar restricted pattern also prevailed in the two sidelines, showing an engagement of groups C E17–18 and G, respectively Though a loss of one G chromosome was seen in two of the four abnormal stemlines, this feature can hardly be regarded as such a significant deviation as in the meningomas Deviations in group F, as often observed in the pituitary adenomas, were not seen in any of the karyotyped cells of the neurinomas

Three of the 4 abnormal stemlines among the neurinomas had one or several markers The frequency of stemlines with markers in the total material of neurinomas was comparable to that observed in the meningomas but it was much higher than that found in the pituitary adenomas This difference might be due to an earlier detection of the adenomas, shortening the time usually available for a structural remodelling of the karyotype

The origin of the markers in each neurinoma was discussed in the previous sections This question is further elucidated by the results of a statistical analysis (Table 5), carried out in accordance with principles earlier outlined by *Levan* (1966) Thus, the neurinomas with markers showed higher negative values (less chromosomes than expected) in groups B, C and E17–18, in comparison with the correspondent figures in the total material, suggesting that these groups were the ones especially involved in the structural rearrangements Conversely, there were no indications that group G, as a rule, participated in the formation of markers, this was a conclusion opposite to that previously drawn in the analysis of the structural variation in the meningomas (*Mark* 1970 a) In comparison with the latter tumour type and the pituitary adenomas, the neurinomas also showed a different representation in several of the ordinary chromosome groups, the most obvious differencies being the positive values for all A chromosomes, the negative values in groups B and C, and the positive value in group D The last two features are particularly unusual findings, as originally shown by *van Steenis* (1966) and by *Levan* (1966), and as previously discussed in the study of malignant gliomas in adults (*Mark* 1971 c) On the basis of the above mentioned features, it can be concluded that chromosomal abnormalities are fairly common in at least three different types of benign, human tumours in the nervous system, the menin gomas, the pituitary adenomas and the neurinomas Furthermore, in each of these tumour types the karyological pattern appears to be different from that in the others

In addition, Table 5 shows the average findings in the 27 karyotyped cells in the modal region of the neurosarcoma Significant deviations from zero (values higher than 0 5) were found in all the ordinary chro mosome groups In comparison with the neurinomas there were many deviating fea tures, for instance the positive value in group B and the negative value in group D It should be noticed, however, that except for the chromosome type markers the highest value was found in group C, and this value was negative, as in the neurinomas

The number of markers in the neurosar coma, on an average 13 7 in the modal cells,

is the highest frequency hitherto encountered in studies comprising more than one hundred primary and secondary neoplasms in the nervous system in man, and it is probably a record notation for all human tumours so far studied (*Sandberg et al* 1967) At first the high frequency of markers was suspected to be due to a previous treatment with X-rays or cytostatics, which incidentally had not been recorded In retrospect, however, this possibility could be excluded Then, the only explanation that can be put forward for the structural variability, is an early formation of highly unstable ring chromosomes or dicentrics carrying genes of great importance for the viability, and consequently persisting in the tumour cells

It could not be proved that the present neurosarcoma had developed from a preexisting neurinoma This assumption, however, seemed highly probable in view of the opinion generally held that all neurosarcomas in patients with von Recklinghausen's disease develop from neurinomas (and not *de novo*), and that the risk of a malignant degeneration is considerable (the incidence among recognized cases being estimated as ranging from between 8 and 15 per cent)

None of the present neurinomas showed any signs of a malignant degeneration Thus, the results did not support the idea advanced by *Benedict et al* (1970), that the occurrence of especially structural, abnormalities in a benign tumour should arise the suspicion of an actual or an approaching malignant transformation

In meningomas (*Singer & Zang* 1970) pituitary adenomas (*Mark* 1971 b) and in gliomas in adults (*Mark* 1971 c), it was possible to demonstrate a correlation between the chromosomal findings and one or several clinico pathological parameters In this respect the results were negative in the present series of neurinomas, but the number of tumours is comparatively small and further cases are necessary to clarify these intriguing questions

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The present investigation was supported by *grants from the Swedish Cancer Society, the University of Lund*, and from *John and Augusta Persson s foundation for medical researches*

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Acta path microbiol scand Section A 80, 71 83, 1972

THE ULTRASTRUCTURE OF MEGAKARYOCYTES IN POLYCYTHAEMIA VERA AND CHRONIC GRANULOCYTIC LEUKAEMIA

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Megakaryocytes from patients with normal haematopoiesis, patients with polycythaemia vera (PCV) and patients with chronic granulocytic leukaemia (CGL) were examined in the electron microscope In addition to the usual cytoplasmic organelles, a regular finding in normal megakaryocytes was the presence of specific granules The normal megakaryocytes were also characterized by the presence of the platelet demarcation system The first evidence of this system was small vesicles, which increased in number, became elongated, and formed demarcation tubules and membranes, delimiting the future platelets Finally, the platelets were demarcated After platelet production was completed, the end result was a naked nucleus almost completely devoid of cytoplasm Megakaryocytes from patients with PCV differed from normal megakaryocytes in certain aspects They could show hypertrophy of both nucleus and cytoplasm, as well as an increase in the amount of ribosomes Rough surfaced endoplasmic reticulum, which was not a regular finding in normal cells, appeared in most megakaryocytes from patients with PCV Some cells appeared abnormal in that they showed no signs of differentiation towards thrombopoietic activity although the cytoplasm was abundant In occasional cells, tubular like double membrane limited profiles enclosing stranded material were found The megakaryocytes from patients with CGL were generally hypotrophic but had a well developed platelet demarcation system In two patients with CGL, defective development of the demarcation system was encountered in some cells These cells showed actual demarcation of platelets in spite of very sparse development of the demarcation system

The significance of the megakaryocytic proliferation in various myeloproliferative disorders is not fully understood Most patients with polycythaemia vera (PCV) and chronic granulocytic leukaemia (CGL) have a very active thrombopoiesis and a marked thrombocytosis The megakaryocytes from the bone marrow in the two conditions show characteristic changes In PCV there is a great variation in the size of the cells with many obviously hypertrophic megakaryocytes, whereas in CGL they are regularly hypotrophic (*Franzen et* al 1961) It is not known whether this megakaryocytic hyperplasia reflects a neoplastic proliferation or a reactive response to some unknown stimulus In an attempt at increasing our understanding of the megakaryocytic proliferation in PCV and CGL the ultrastructure of these cells has been examined

Received 16 vi 71

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MATERIAL AND METHODS

Bone marrow material from 6 patients with PCV, 5 patients with CGL and 4 control patients, was investigated ultrastructurally Some data for the patients are compiled *in table* 1 The control patients had normal thrombopoiesis, as judged by the peripheral blood values and ordinary cytological and histological examination of the bone marrow In 4 patients with PCV, the bone marrow was taken when the patients were first admitted to the clinic before any specific treatment had been initiated One patient, no 9, had been exposed to treatment with 5 mC ^{32}P five times, the last time one year before bone marrow material was removed for electron microscopy In one patient, no 6, *venesection had been* performed four times prior to the time when the marrow was taken for electron microscopy The CGL bone marrow material was taken from the patients before specific treatment had been initiated The bone marrow *from all patients with PCV and CGL had* increased numbers of megakaryocytes when examined cytologically and histologically In the marrow from the PCV patients the megakaryocytes were highly pleomorphic with several hypertrophic cells (Fig 1) In the marrow specimens from the cases with CGL, the appearance of the megakaryocytes was *more uniform* with most cells being hypotrophic (Fig 2)

Preparation of Bone Marrow for Electron Microscopy

The bone marrow was removed by ordinary sternal puncture employing the Franzen Kifa instrument Immediately after removal, small pieces of the marrow were fixed in 4 per cent glutaralde [illegible] in cacodylate buffer at pH 7 2 for 3 12 hours then postfixed for 1 hour in 1 per cent osmic [illegible] in veronal buffer at pH 7 2 The specimens [illegible] embedded in epon and then sectioned at approximately 400 Å with a LKB Ultratome The sections were stained with 4 per cent uranyl acetate for 1 hour at 60° C, then with lead citrate (*Reynolds* 1963) and examined in a Siemens Elmiscope I A

Preparation of Bone Marrow for Cytological and Histological Examination

Pieces of marrow were also immediately smeared on ordinary glass slides and stained with May Grunewald Giemsa for cytological examination Other pieces of marrows were fixed in Stieves fluid (mercuric chloride formalin and glacial acetic acid), dehydrated and embedded in paraffin Sections at 4μ were stained with haematoxylin eosin for examination by light microscopy

RESULTS

The Ultrastructure of Megakaryocytes from patients with normal haematopoiesis

Sixty megakaryocytes from the 4 haematologically normal subjects were analysed ultrastructurally The findings were largely in agreement with earlier observations on rat and mouse megakaryocytes (*Gautier et al* 1963, *Hahn & Baker* 1964, *Paulus* 1970 *Yamada* 1957)

Nucleus

The size of the nucleus varied greatly from cell to cell In cells with a well differentiated cytoplasm, only one or two nuclear segments were sometimes found, even after sectioning through the whole cell In other cells, the nucleus appeared as a solid mass or as segments of a multilobulated nucleus In the majority of nuclei, the chromatin was finally dispersed throughout the nucleus with a slight condensation along the nuclear membrane (Figs 3 and 4) More pronounced conden

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Fig 1 Section of paraffin embedded bone marrow from a patient with PCV The number of megakaryocytes is increased and many of them are markedly hypertrophic Haematoxylin eosin Magnification 360 ×

Fig 2 Section of paraffin embedded bone marrow

one nuclear lobe Haematoxylin eosin Magnification 360 ×

Fig 3 Immature megakaryocyte (megakaryoblast) from control patient number 1 The large, lobulated nucleus contains finely dispersed chromatin and several nucleoli The cytoplasm is scanty Ribosomes are frequent in part attached to endoplasmic reticulum A group of vesicles is seen in the right lower portion of the cell but demarcation tubules and membranes are absent There are no signs of delineation or delimiting of thrombocytes in the cytoplasm

Fig 4 Mature megakaryocyte from control patient number 1 The nucleus contains finely dispersed chromatin and a few nucleoli There is a moderate amount of cytoplasm the appearance of which is dominated by an abundance of demarcation tubules and membranes delimiting the future thrombocytes indicated by arrows


2
10 μ
4
10 μ

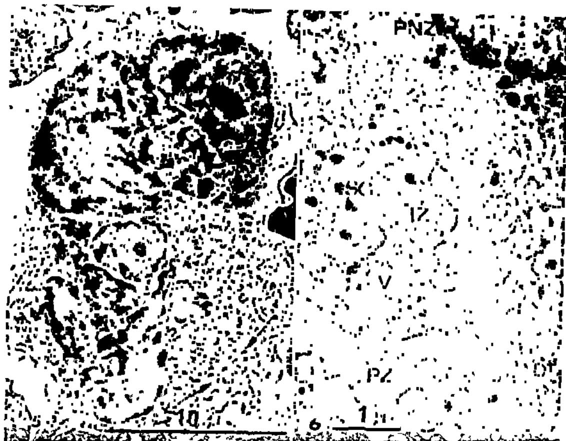


Fig 5 Mature megakaryocyte from control patient number 2 The nucleus shows marked condensation of the chromatin The cytoplasm is scanty *In the left part of the cell almost all cytoplasm is* lost due to completed thrombopoiesis, whereas active delimiting of thrombocytes is still evident in the remaining cytoplasm, indicated by arrows

Fig 6 Detail of cytoplasm of mature megakaryocyte from
(PNZ) *contains only occasional vesicles and tubules Th*
part of the cytoplasm Here all the cell organelles can be
V) and demarcation tubules (DT) The peripheral zone (PZ) occupies the outer rim of the cytoplasm
contains only few cell organelles

sation of the chromatin occurred only in cells where the cytoplasm exhibited signs of completed *thrombopoiesis* (Fig 5) *One to several* nucleoli were observed in almost all cells, and only those cells with pronounced condensation of the chromatin were devoid of nucleoli The nuclear envelope usually appeared to consist of two membranes

Cytoplasm

Zonation As has been pointed out earlier, the cytoplasm can be divided ultrastructurally into *three different zones* perinuclear, intermediate, and peripheral (*Gautier et al* 1963 *Hahn & Baker* 1964, *Yamada* 1957) (Fig 6) The perinuclear zone contained ribosomes, *mitochondria and a prominent Golgi* complex which extended into the intermediate zone (Fig 7) Few specific granules were visible Vesicles and demarcation membranes were absent or very sparse in the perinuclear area There was no distinct boundary between the perinuclear and intermediate zone

The intermediate zone constituted the major part of the cytoplasm (Fig 6) All the cell organelles could be found here, and it is in this zone that the demarcation of thrombocytes takes place (Figs 4 and 5)



Fig 7 Golgi complex (G) in a megakaryocyte from control patient number 4 Adjacent to the Golgi complex several vesicles of varying size appear. Mitochondria indicated by M

In the peripheral zone, cytoplasmic organelles were very sparse (Fig 6). The width varied, but was usually thin, and always occupied a much smaller part of the cytoplasm than did the intermediate zone

Cytoplasmic Organelles

Vesicles and demarcation membranes and tubules These were present in varying amounts, depending on the stage of maturation as accounted for below

Specific granules Lysosomes with a diameter measuring 0,1 to 0,4μ were present in large amounts in all phases of maturation (Fig 6) They were surrounded by a single membrane and had a moderately electron dense interior that sometimes showed a darker centre (Fig 9)

Mitochondria These were also present in all maturation phases They were most numerous in the inner part of the intermediate zone and in the perinuclear zone (Fig 7) They were round, elongated, and sometimes long and slender, with a diameter of about 0 2–0,4μ They were often electron dense and were therefore occasionally difficult to differentiate from the specific granules.

Ribosomes These were present in moderate amounts in the immature cells and were somewhat more sparse in the cells that were considered more differentiated, as judged by the appearance of the demarcation membranes Polyribosomes were seldom seen in normal megakaryocytes

Endoplasmic reticulum Rough surfaced or smooth endoplasmic reticulum was seen in some cells, but was most abundant in the immature cells Although it occurred in all zones, it was found mostly near the nucleus or oriented to the periphery

Golgi complex The Golgi complex was usually well-developed (Fig 7) It was located in the perinuclear zone reaching into the intermediate zone The vesicles and demarcation tubules appeared to emanate from the Golgi complex

Phases of maturation Differentiation of the megakaryocytes could be followed by the characteristic changes in the appearance of cytoplasmic vesicles and demarcation tubules and membranes The development of the



Fig 8 Mature megakaryocyte from PCV patient number 5 The nucleus (N) is huge and highly lobulated The chromatin is finely dispersed and contains a few nucleoli (n). The cytoplasm is abundant and contains large amounts of demarcation tubules and moderate amounts of specific granules The scarcity of cytoplasmic organelles in the pheripheral zone (PZ) is striking

megakaryoblast to the mature megakaryocyte and finally, after completed thrombopoiesis, a pycnotic cell, is not a stepwise develop- t, but involves a gradual alteration in the of vesicles and demarcation membranes and tubules In the most immature cells few vesicles and no demarcation membranes were observed A sign of differentiation was the increasing number of vesicles (Figs 3 and 7) The size of the vesicles was variable but they generally measured 0,3 to 1,0μ and were surrounded by a single membrane The first sign of delineation of the future platelet was the elongation of the vesicles forming demarcation tubules, which would later on appear as long rows of parallel membranes, *i e* the demarcation membranes In the highly-differentiated cells, the cytoplasm was dominated by these tubules and membranes (Figs 4 and 6) Finally, there was the actual demarcation and liberation of platelets At this stage the nucleus showed condensation of its chromatin (Fig 5)

The Ultrastructure of Megakaryocytes from patients with PCV

Ninety-six megakaryocytes from 6 patients with PCV were examined ultrastructurally All patients had some variations in the ultrastructural appearance of the megakaryocytes In most cells there were slight deviations from the normal whereas some cells were obviously abnormal

Nucleus

The size of the nucleus was variable Some cells (12 of 96) obtained only one or two

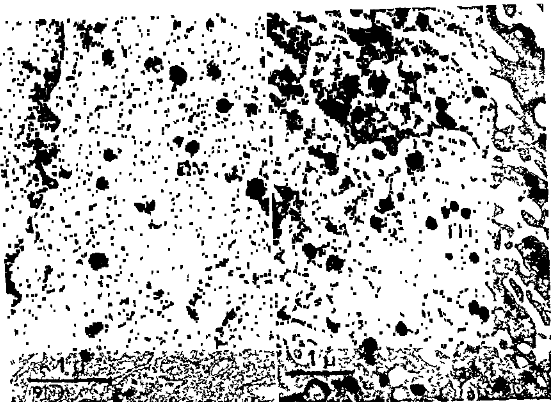


Fig 9 Detail of mature megakaryocyte from PCV patient number 5 Large numbers of specific granules and demarcation membranes (DM) can be seen

Fig 10 Detail of mature megakaryocyte from PCV patient number 10 Large numbers of specific granules and demarcation tubules are present, and actual delimiting of thrombocytes (TH) can be seen

nuclear lobes, whereas others (25 of 96) exhibited huge nuclei with marked lobulation (Fig 8). In the majority of cells (59 of 96), the size and lobulation of the nucleus corresponded to that of normal megakaryocytes The chromatin was almost always finely dispersed and only in a few cells, where the appearance of the cytoplasm indicated that the thrombopoiesis was almost complete, did the chromatin show marked condensation Most nuclei contained one to several nucleoli The nuclear membrane showed no deviation from the normal picture

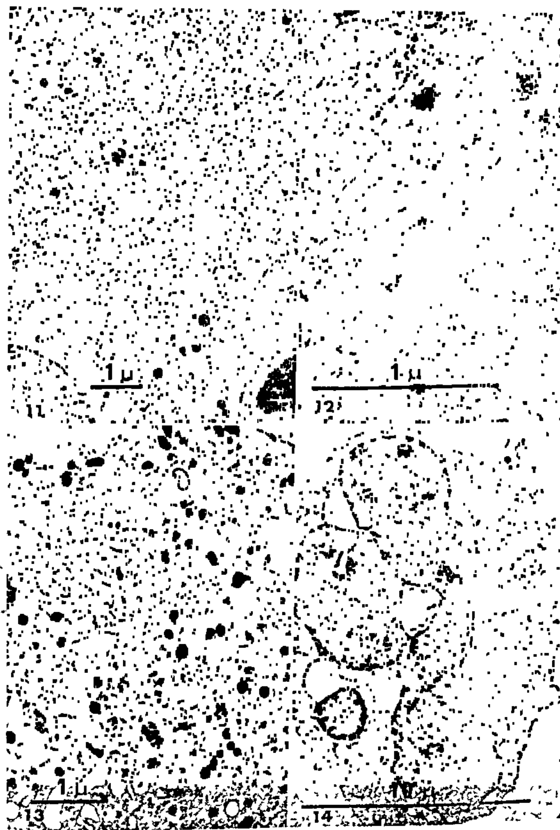
Cytoplasm

Size The size of the cytoplasm also varied A few very immature cells and cells with almost completed thrombopoiesis had sparse cytoplasm In 45 cells the amount of cytoplasm corresponded to that of normal megakaryocytes, whereas 44 cells were classified as definitely hypertrophic The marrow sample from case number 9, which had been exposed to treatment with ^{32}P, differed by having only one hypertrophic megakaryocyte, whereas 3 of 12 cells were definitely smaller than usual, one of those being immature and the other two well-differentiated

Zonation As in the normal cases, the cytoplasm could be divided into a perinuclear, intermediate, and peripheral zone In cases with enlarged cytoplasm, the intermediate zone was broadened

Cytoplasmic Organelles

Demarcation vesicles, tubules and membranes In the majority of cases, the vesicles, demarcation tubules and membranes ap-


1 µ
11
1 µ
12
1 µ
13
1 µ
14

peared as in megakaryocytes from normal cases (Figs 8, 9 and 10), however, 7 cells from cases number 5 and 6 differed characteristically from the other cells All these 'abnormal' cells had abundant cytoplasm but lacked vesicles and demarcation tubules and membranes to give the cytoplasm an immature, undifferentiated appearance (Fig 11)

In four cells from case number 5, several tubular like double membrane limited profiles enclosing stranded material were found, in addition to normal demarcation tubules and membranes (Fig 12) Similar profiles have not been found in megakaryocytes from the control patients or patients with CGL It was not possible to determine the significance of these double membrane limited profiles

Granules In the cells devoid of demarcation system that were mentioned above, the specific granules were also somewhat sparse, although not absent Except for these cases, the granulation of the cytoplasm appeared as in normal cells (Figs 9 and 10)

Fig 11 Detail of cytoplasm of megakaryocyte from PCV patient number 5 The cytoplasm is abundant but no vesicles or demarcation tubules or membranes are present There are large numbers of ribosomes and polyribosomes and rough surfaced endoplasmic reticulum is seen

Fig 12 Detail of cytoplasm of megakaryocyte from PCV patient number 5 Tubular like double membrane limited profiles enclosing stranded material can be seen

Fig 13 Detail of cytoplasm of megakaryocyte from PCV patient number 7 There is an abundance of rough surfaced endoplasmic reticulum and very moderate occurrence of vesicles The specific granules are very frequent

Fig 14 Megakaryocyte from CGL patient number 11 The nucleus has a moderate size and finely dispersed chromatin The cytoplasm is sparse There is a very moderate number of ribosomes mitochondria and specific granules. Demarcation tubules and membranes are absent but some vesicles are distributed evenly in the whole cytoplasm

Mitochondria In 15 of 96 cells a marked increase in the number of mitochondria was seen The remaining cells contained about the same number of mitochondria as the megakaryocytes of the control patients. Their rounded or elongated shape and their size were as shapes and sizes in normal cases

Ribosomes In 29 of the cells the ribosomes were present in greater than normal amounts, some being grouped as polyribosomes In 9 cells there was a considerable increase in ribosomes and in those cases they occurred mostly as polyribosomes All the previously mentioned immature cells devoid of demarcation membranes and tubules belonged to this group (Fig 11). The distribution of the ribosomes was similar to that of megakaryocytes from the control cases, with the peripheral zones usually devoid of ribosomes

Endoplasmic reticulum Rough surfaced endoplasmic reticulum infrequently seen in normal cases, was found in almost every cell from patients with PCV It was present in a substantial amount in some cells, especially those cells with abnormal differentiation mentioned above (Fig 11) It occurred mostly near the perinuclear area and in the outer part of the intermediate zone (Fig 13)

Golgi complex The Golgi complex did not differ in any particular aspects from that seen in control patients megakaryocytes

In summary, megakaryocytes from patients with PCV differed ultrastructurally from megakaryocytes from haematologically normal patients in the following aspects megakaryocytes from patients with PCV could show hypertrophy of both nucleus and cytoplasm the amount of ribosomes was often increased and in some cases considerably the number of mitochondria could be increased rough surfaced endoplasmic reticulum appeared in some cells and in some cases in large amounts Seven out of 96 cells appeared definitely abnormal by showing no sign of differentiation towards thrombopoietic activity although their cytoplasm was abundant In 4 cells from one patient tubular like double membrane limited profiles enclosing stranded material were found

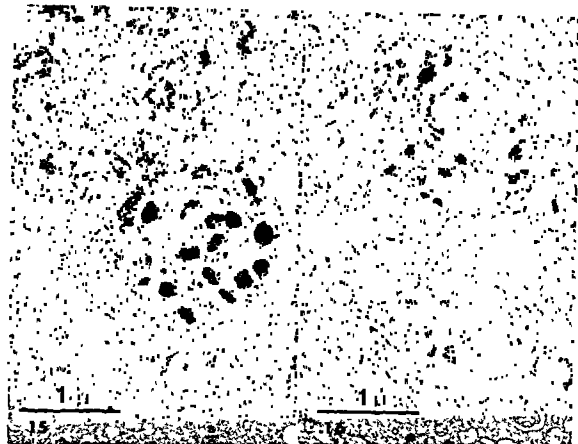


Fig 15 Higher magnification of the megakaryocyte in Fig 14 In spite of the absence of the normal thrombocyte demarcation delineation of a thrombocyte is clearly evident

Fig 16 Cytoplasm of megakaryocyte from CGL patient number 14 Moderate amounts of vesicles are present, but very few demarcation tubules In spite of this there is actual demarcation of two thrombocytes

The Ultrastructure of Megakaryocytes from Patients with CGL

Ninety four megakaryocytes from 5 patients were examined

Nucleus

Sixty-four of the 94 cells studied contained only one or two nuclear segments, whereas in 30 cells three or more segments were observed Usually their size was small, however, (Fig 14) and only a few nuclei had the appearance of the nuclei in megakaryocytes from normal cases Unlike nuclei from the megakaryocytes of patients with PCV no hypertrophic nuclei were observed

The nuclear chromatin was usually finely dispersed as in normal and polycythemic cases, and often one or two nucleoli were seen Only in those cells showing signs of completed thrombopoiesis there was more pronounced condensation of the chromatin The nuclear membrane appeared normal

Cytoplasm

Size In 59 out of the 94 cells the amount of cytoplasm was considered to be less than in normal megakaryocytes (Fig 14) and in 35 cells there was approximately the same amount of cytoplasm as in normal megakaryocytes

Zonation No deviation from the normal zonation was seen in megakaryocytes from patients with CGL

TABLE 1 *Survey of Peripheral Blood Values*

	Patient no	Sex	Age (years)	Hb %	White cells/ mm^3	Thrombocytes/ mm^3
Normal	1	♂	69	15,5	19 000	195,000
	2	♂	34	12,7	5 000	190 000
	3	♂	46	12,4	4 500	140 000
	4	♀	61	12 0	8 000	240 000
PCV	5	♀	75	16 8	23 500	780 000
	6	♂	68	16 2	17 900	580 000
	7	♀	66	13,3	12.300	800 000
	8	♀	75	17,2	70 000	150 000
	9	♀	70	11,7	12 500	160 000
	10	♀	57	19,3	15 700	470 000
CGL	11	♂	68	9 0	263 000	242 000
	12	♀	41	9,0	164 000	320 000
	13	♀	57	14 2	62 000	240 000
	14	♂	64	8,9	60 400	315 000
	15	♂	38	13 9	8 400	200 000

Cytoplasmic Organelles

Vesicles, demarcation tubules and membranes In 52 of 82 cells from 4 of the patients with CGL, the demarcation system was well developed and many cells showed signs of active thrombopoiesis The remaining 30 cells showed less development of vesicles and sparse occurrence of tubules and membranes, consistent with a lower degree of cytoplasmic differentiation than in the other cells

In patient number 11, 8 cells showed a defective development of their demarcation systems The cytoplasm of 2 of these cells contained neither vesicles nor tubules or membranes, but had no rough surfaced endoplasmic reticulum and the amount of ribosomes was moderate as in normal mature cells Specific granules were present in normal amounts in these cells Three of the cells contained a small number of vesicles but no tubules or membranes in an otherwise mature cytoplasm (Figs 14 and 15) The remaining 5 cells contained small numbers of vesicles, tubules and membranes Despite the sparseness of demarcation systems, there was, nevertheless, an unequivocal demarcation of platelets within the cytoplasm of these cells As is evident from table 1, this patient did not present with a thrombocytosis, but had 242 000 thrombocytes per mm^3 in the peripheral blood

Similar ultrastructural findings were seen in 3 cells from patient number 14 In spite of their poorly developed demarcation tubules and membranes, these cells showed actual demarcation of platelets within their cytoplasm (Fig 16)

Specific granules Specific granules occurred in normal amounts and had a normal appearance

Mitochondria The number and appearance of the mitochondria did not deviate from the findings in normal megakaryocytes No definitely hypertrophic mitochondria were found

Ribosomes Ribosomes occurred in moderate amounts in all cells and polyribosomes were seldom encountered (Fig 15)

Endoplasmic reticulum Rough surfaced endoplasmic reticulum was found in 12 cells It was very sparse, however, and was never similar to that found in some cells from patients with PCV

Golgi complex The appearance of the

Golgi complex did not deviate in any particular way from that seen in normal megakaryocytes

In summary, the megakaryocytes from patients with CGL were generally hypotrophic, but usually showed a well differentiated cytoplasm, with a well developed demarcation system, and active thrombopoiesis In the megakaryocytes from two patients, defective development of the demarcation system was evident These cells showed actual demarcation of platelets in spite of a very sparsely developed demarcation system

DISCUSSION

Patients with PCV and CGL usually show a characteristic proliferation of the megakaryocytic cell series in their bone marrow The present investigations revealed that the majority of the megakaryocytes in both diseases have a well developed platelet demarcation system and are active platelet producers The megakaryocytic hyperplasia in the bone marrow does therefore not seem to constitute a reactive hyperplasia to compensate for a defect in the function of the megakaryocytes In PCV and CGL the megakaryocytic hyperplasia is usually also associated with a peripheral thrombocytosis Usually the thrombocytes from patients with PCV and CGL have [illegible]d function and one serious complica[illegible] is the development of thromboses The [illegible] granules, which constitute the lysosomal component of the cytoplasm of the megakaryocytes, have not shown any obvious abnormalities in the present study These structures are supposed to carry coagulation factors (1 and 3) and the lysosomal enzymes (*Schultz* 1968) The normal occurrence of the specific granules is thus in good agreement with the conception of preserved normal function of the thrombocytes produced by the megakaryocytes from patients with PCV and CGL

Behnke found the demarcation system of rat megakaryocytes to be derived from the plasma membrane and the cavities of the demarcation tubules to be continuous with the extracellular space (*Behnke* 1968) In the present and earlier studies (*Schultz* 1968) the platelet demarcation system in the cytoplasm of megakaryocytes appears to emanate from small vesicles formed adjacent to the Golgi complex Earlier studies (*Schultz* 1968, *Yamada* 1957) and the present work have demonstrated a very regular development of the demarcation tubules and membranes in megakaryocytes from normal subjects through characteristic maturation stages Cytoplasmic maturation and increase in cytoplasmic volume did not occur without concomitant development of the platelet demarcation system in normal megakaryocytes The abnormalities encountered in megakaryocytes from some of the patients with PCV and CGL were recognized mainly as a failure to normal development of the platelet demarcation system

An other study on the ploidization of megakaryocytes showed great variations in the DNA histograms of PCV megakaryocytes and a shift to lower ploidy levels in CGL megakaryocytes (*Lagerlöf* 1971) The electron microscopic study does not allow determination of the exact ploidy level of each megakaryocyte, but the size of the nucleus gives some information A small only slightly lobulated nucleus, which could be consistent with a low ploidy level, could be found in cells with a mature cytoplasm and a well developed platelet demarcation system This is in agreement with the findings in guinea pig megakaryocytes (*Paulus* 1970)

Abnormalities of the cytoplasmic differentiation have been encountered only in relatively few megakaryocytes from patients with PCV or CGL These abnormalities might well reflect an isolated disturbance of the differentiation process in these cells and does not convincingly indicate that the cells are neoplastic

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This work was supported by grants from the *Swedish Cancer Society* the *Swedish Medical Research Council* and funds from the *Karolinska Institutet*

Efficient technical assistance by Miss *Maj Andersson* is gratefully acknowledged

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STUDIES ON THE LYMPHOCYTES IN THE INTESTINAL EPITHELIUM OF THE CHICKEN

1 *Ontogeny*

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The occurrence of lymphocytes within the ileal epithelium was studied in newly hatched, 6 day-old, 7 and 9 week-old and 18 month old chickens The number of epithelial lymphocytes (theliolymphocytes) increased markedly with age The specific localization of the epithelial lymphocytes differed from that in other species described, i e rodents and man, in the respect that a larger proportion of the lymphocytes were distributed towards the surface of the epithelium, especially in the 6 day old chickens Globule leucocytes in the epithelium were proportional to the number of epithelial lymphocytes and their number increased with age just as that of the epithelial lymphocytes The increase in the number of epithelial lymphocytes with age was considered to be connected with the peripheraliza tion of the second level lymphoid tissues and the maturation of the immune response

The occurrence of lymphocytes within the intestinal epithelium was described more than a hundred years ago Several attempts to clarify their function at this site have been made (review by *Wolf Heidegger* (33)), but our knowledge is still limited Recent experimental studies in man and mammals indicate that the lymphocytes migrate from the lamina propria into the epithelium and back again (22) and there seems to be little evidence favouring the old hypothesis of a trans epithelial migration to the intestinal lumen (26)

Phylogenetically the diffuse infiltration of lymphocytes in the gut epithelium appears prior to aggregates of gut associated lymphoid tissue, and it is interesting that in some fishes the lymphocytes amount to 40–50 per cent of the cells in the gut epithelium (11) In mice the number of epithelial lymphocytes increases from birth to adulthood There is a striking and rapid increase during the third week of life, parallel with the development of the immune response (10)

From immunobiological points of view the lymphocytes in the intestinal epithelium of the chicken are of great interest Neonatal bursectomy depresses the primary antibody response, but only temporarily It has been suggested that the restitution may be related to the lymphoid tissue associated with the digestive tract (17) A theory has also been advanced suggesting that the intestinal epithelium or specialized parts of it may have a bursal function (8) Neonatal bursectomy or thymectomy producing different kinds of immunological deficiencies (4, 12, 29), may

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provide models broadening our knowledge about the status of the epithelial lymphocytes

The aim of this investigation was to study the occurrence of the epithelial lymphocytes, here called theliolymphocytes, in different age groups of the chicken Special attention will be paid to the localization of the theliolymphocytes within different regions of the villus and at various levels of the epithelium

MATERIAL AND METHODS

Histological slides from chicken ileum of five different age groups were studied The relative numbers of theliolymphocytes in the top and base regions of the villous epithelium (Fig 1) and at different epithelial levels (Fig 2) were recorded

Animals

Newly hatched Seven male white Leghorn hybrids of the Babcock B-300 strain, obtained from a local hatchery on the morning of the 21st day of incubation were used Their mean weight was 37 grams (range 34-40 grams) and they were killed on the same day between noon and 2 p m

6 day old Eight male chickens from the same hatch and strain as above were used They were marked with wing clips on the day of hatching and kept in a heated cage until they were killed six days later Their mean weight was then 60 grams (range 56-65 grams)

7-week old Ten newly hatched male chickens of the same strain and source as above were kept in our animal quarters until 7 weeks old Their mean weight when killed was 370 grams (range 320-420 grams)

9 week-old Six cockerels of the same strain and source as above with a mean weight of 700 grams (range 675 770 grams) were examined

18 month-old Six hens, weighing about two kilograms (range 1 9 2 2 kilograms) were kept in our animal quarters for four weeks before being killed at the age of 18 months They were supplied by the Department of Animal Husbandry Agricultural College, Ultuna, Uppsala, Sweden and were white Leghorn hybrids of the De Kalb strain

All the animals of the above four age groups received the same food and were killed at the same time of day, between noon and 2 p m

Biopsy and Histological Techniques

All animals were killed by air emboli introduced into a wing vein Biopsies were taken from the small intestine proximal to the ileo colic caecal junction In the neonatal chickens the distance to this junction was 1 cm and in the 7-week-old and 18 month-old animals it was 3 cm. The specimens were cut along the mesentery and pinned on to a piece of cork, and subsequently fixed in carbonate-buffered 4 per cent formalin overnight The 18-month-old animals were given 40 mg papaverine intravenously just before they were killed, in order to reduce the muscular activity of the gastrointestinal tract The tissue specimens were embedded in paraffin after dehydration in alcohol and xylene, sectioned at 5 microns and stained in Mayer s haemalum and eosin.

Microscopic Evaluation of the Slides

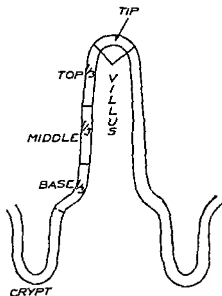
The lining epithelium of longitudinally sectioned villi was subdivided into three regions top, middle, and base (Fig 1) The extreme tips of the villi were excluded At least 1000 epithelial cell nuclei were counted in the top region and the same number in the base region of the villi in every animal The number of "infiltrating" cells within these areas was recorded, as well as their locations at different levels of the epithelium (Fig 2) An oil immersion objective giving a final magnification of × 1000 was used

Cell Differentiation Criteria

The epithelial columnar cell has an oval nucleus with a loose chromatin pattern There are sometimes one or two nucleoli The cytoplasm is slightly eosinophilic The goblet cell has a characteristic appearance with a dense elongated or triangular nucleus which is often pushed towards the basement membrane by the pale, slightly basophilic mucus in the cytoplasm The small, round or irregular nucleus of the lymphocyte is fairly dense Its cytoplasm is scanty and sometimes a pale halo can be seen around the nucleus The eosinophilic leucocyte mostly has a bilobar nucleus The granules have a characteristic bright-reddish colour The nucleus of the globule leucocyte is coarse and indented and is similar to that of the lymphocyte The cytoplasm contains some eosinophilic globules, which are larger and appear more yellow than the granules of the eosinophils These cells are probably the 'Schollen leukozyten' described by *Weill* (30)

RESULTS

In each of the top and base regions of the villous epithelium (Fig 1) the area occupied by 1000 epithelial columnar cells was examined The results from the differential cells counts in various parts of the epithelium are given in Table 1 Significant differences were found between the five age groups (Table 1),



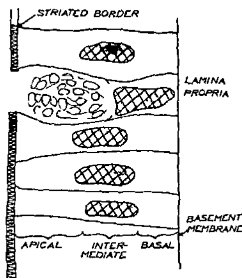


Fig 1 The figure shows the regions of the villous epithelium The top and base regions only are counted in this investigation

Fig 2 The three zones within the intestinal epithelium apical intermediate and basal denoting the location of a lymphocyte vis à vis the row of epithelial cell nuclei

and the number of theliolymphocytes increased with age The relative number of theliolymphocytes in the top and base regions was about the same in all age groups except in the 6 day old group where the base region ($P < 01$) and the 9 week old group where the top region ($P < 001$) showed significantly larger numbers

The distributions of the theliolymphocytes within the epithelium are also presented in Table 1 Most of them were localized in the basal zone of the epithelium between the row of epithelial cell nuclei and the basement membrane About 20 per cent of the theliolymphocytes or fewer were seen in the apical zone of the epithelium except for the base region of the 6 day old chickens, where as many as 43 per cent had adopted a position near the epithelial surface In none of the animal groups was the number of theliolymphocytes in the apical zones of the top region significantly higher than in the corresponding epithelial area of the base region The theliolymphocytes in the apical zone of the epithelium were not observed in the striated border but sometimes just below In the 9 week old group with more theliolymphocytes in the top than in the base region the increase was confined to the basal and intermediate zones

Mitotic figures were frequently seen in the crypts but not in the villous epithelium

In addition to lymphocytes also eosinophilic and pseudo eosinophilic leucocytes and globule leucocytes were seen in the epithelium The neonatal group showed very few eosinophilic and globule leucocytes In the 6 day-old chickens the globule leucocytes were still very few but the eosinophils were as numerous as $3\,2 \pm 0\,8$ (mean $\pm$ standard error of the mean) in the top region and $0\,9 \pm 0\,3$ in the base region counted per 1000 epithelial columnar cells In the older groups the globule leucocytes were more

TABLE 1 *Number of Theliolymphocytes per 1000 Epithelial Columnar Cells in the Top and Base Regions of the Villi*

Age group	Region	Basal	Localization Intermediate	Apical	Total	Probability of differe between consecutive age gro Top regions	Base re
Neonatal n = 7	Base	7 0± 1 1	1 1±0 2	1 8±0 4	10 0± 1 5		
	Top	8 1± 1 9	0 7±0 4	2 1±0 6	10 9± 2 4	P< 001	P< 0
6-day-old n = 8	Top	22 9± 2 3	2 0±0 6 (P< 02)	5 5±0 8 (P< 005)	30 4± 2 7 (P< 01)		
	Base	20 1± 2 4	5 2±0 9	19 2±2 7	44 4± 3 7	P< 001	P< 0
7 week old n = 10	Top	46 9± 3 1	5 8±1 2	10 0±1 7	62 8± 4 3		
	Base	51 1± 3 3	5 8±0 6	12 5±1 4	69 4± 3 8	P< 001	P< (
9 week old n = 6	Top	91 7± 5 5 (P< 05)	24 2±2 7	31 7±2 7	147 5± 4 7 (P< 001)		
	Base	74 8± 2 4	17 7±1 5	30 0±1 2	122 5± 2 2	P< 02	P<
18 month-old n 6	Top	81 3±11 4	9 7±1 2	9 5±2 0	100 3±11 0		
	Base	86 6±10 6	9 0±1 0	7 5±1 6	103 1±10 3		

The figures are given as mean ± standard error of the mean The terms basal, intermediate and apical designate the localization of the lymphocyte in relation to the row of epithelial cell nuclei (Fig 2) Significance levels of the differences were calculated with the Student s *t* test or with the approximation of the Behrens Fisher test according to Cochran

prominent and as many as 18 ± 3 and 19 ± 2 per 1000 epithelial cells were recorded in the top and basal regions of the 7-week-old chickens The hens showed approximately the same number 25 ± 5 and 19 ± 2 in the corresponding areas, while the 9 week-old cockerels presented 49 ± 5 and 31 ± 3 in the two regions The number of globule leucocytes was then almost proportional to the number of theliolymphocytes Only occasional eosinophilic or pseudo eosinophilic leucocytes were found in these older groups

DISCUSSION

The examination of the intestinal epithelium was confined to the top and base regions of the villi since these areas represent morphologically and physiologically different epithelia (23) The crypts were not included in the study because they are not readily sectioned along their entire length and the numerous mitotic figures cannot be classified into different cell lines Within the epithelium the various levels vis a vis the row of epithelial cell nuclei were denoted apical, intermediate and basal (2) in order to determine whether any pattern existed in the theliolymphocyte localization

This quantitative study of the chicken strongly suggests that the number of theliolymphocytes increases with age This correlation is interesting with regard to the peripheralization of the lymphoid tissue in this animal In the peripheral blood the proportion of lymphocytes increases from 25 per cent at hatching to 65–70 per cent ten days later (24) In the spleen and the caecal tonsils the differentiation towards lymphoid organs becomes evident during the first week after hatching The bursa dependent follicles in the spleen and the caecal tonsils do not appear until at the age of 2–3 weeks (7, 18) Some transplantation reactions and antibody production appear during the first week, but the full maturation of the immune response is not attained until later (25) The gradual increase in the number of theliolymphocytes

with age may reflect this peripheralization and the diffuse infiltration of lymphocytes in the gut epithelium could be regarded as a second level lymphoid phenomenon

Theliolymphocytes in the chicken have been observed in the intercellular spaces between the epithelial columnar cells (32) The route of absorbed and digested material from the lumen is supposed to be through the apical part of the absorbing cell to the intercellular spaces and then across the basement membrane to the lymph and blood vessels in the lamina propria (28) The location of the theliolymphocytes in this pathway is suitable for direct contact absorbed antigens Newborn animals and man have been shown to absorb proteins with intact antigenic determinants (3 14) Even later in life proteins may pass from the gut to the serum and urine In rodents and man more than 95 per cent of the theliolymphocytes lie on the basement membrane (22) but in the chicken the lymphocytes are scattered over the epithelium to a greater extent It is interesting that in the base region of the villi the 6 day old chickens have about 40 per cent of the lymphocytes in the apical zone The interpretation of this may be that the lymphocytes accumulate at a place where processed or unprocessed antigens are available and this may be the case in these young chickens which are adapting to the environment

The presence of lymphoid cells in the lamina propria seems to be dependent upon the microbial flora in the intestine Germ free animals have few plasma cells in the lamina propria and feebly developed Peyer s patches (13) Germ free animals when infected normalize their intestinal mucosa within a few days with a rapid increase of the gut associated lymphoid tissue (6) and of the epithelial proliferation rate (1) This physiological inflammation present in all conventional animals is a second level lymphoid phenomenon by definition and the appearance of its morphological characteristics is very well paralleled by the peripheralization of the lymphoid tissue and the appearance of the theliolymphocytes The result from an investigation in germ free and conventional rats reported by *Fichtelius* (9), that the number of theliolymphocytes was about the same in the two animal groups is in accord with previous findings in germ free and normal stock chickens (13) Thus the infiltration of lymphocytes in the intestinal epithelium may be a phenomenon different from the presence of lymphocytes and plasma cells in the lamina propria of the gut

A comment may also be made on the old theory of a trans epithelial migration of the lymphocytes If there were such a migration the result would be an accumulation of lymphocytes in the apical zone of the epithelium in the top region However the proportion of theliolymphocytes was about the same in the top and base regions and with the exception of the 9 week old groups the relative number of theliolymphocytes within the apical zone did not differ between these two regions Although some theliolymphocytes were situated just below the striated border none were observed to pass through These findings constitute evidence opposing the trans migration theory and favouring recent views most clearly expressed by *Fichtelius* (8) and *Meader* & *Landers* (22)

During the last decade our knowledge about the defence of the mucous membranes has been broadened The concept of the IgA system is in the process of elaboration (5 27) A role of the theliolymphocyte as a mediator in this system is as yet speculative There are however some reports suggesting an immunity mediated by antibodies other than the established IgM and IgG since oral immunization with killed *Pasteurella multocida* organisms may produce an active immunity irrespective of negative agglutination and precipitin tests (15 16)

In an extended study of the sheep *Kent* (19) suggests that the globule leucocytes are of lymphocyte origin and destined for the intestinal lumen The number of globule leucocytes in the gut epithelium of germ free chickens is significantly reduced in comparison with the number in normalstock chickens (13) indicating a function in the

mmunological defence of the intestinal nucosa The globule leucocytes have also een observed in the epithelium of the espiratory (32) and genito urinary (33, 48) racts In the present study the occurrence of the globule leucocytes showed the same orrelation with age as that of the thelio-ymphocytes The number of globule leuco cytes was also proportional to the number of *theliolymphocytes* These two findings support the suggestion that globule leucocytes are of lymphocyte origin

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The work was supported by the *Medical Faculty of the University of Uppsala* and the *Swedish Medical Research Council*

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STUDIES ON THE LYMPHOCYTES IN THE INTESTINAL EPITHELIUM OF THE CHICKEN

2 *Kinetics*

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Seven week-old male chickens were injected intravenously with ^{3}H thymidine The presence of labelled lymphocytes in the ileal epithelium was studied by means of autoradiography at various times after injection There was a significant increase in the labelling index, i e the percentage of labelled epithelial lymphocytes, here called theliolymphocytes, between 1 hour and 48 hours after labelling This increase was considered to be due to both mitosis of labelled theliolymphocytes and immigration of labelled lymphocytes from the lamina propria During the following 4 days the labelling index decreased The labelling index in top and base regions of the villi did not differ The labelling indices of the theliolymphocytes 48 hours and 6 days after ^{3}H TdR administration were significantly higher than the percentages of the labelled peripheral blood lymphocytes at corresponding times This finding suggests an accumulation of labelled lymphocytes in the intestinal epithelium. In the top regions of the villi, where most of the absorption from the intestine takes place, there was a significantly higher proportion of labelled theliolymphocytes in the apical zone than in the intermediate and basal zones of the epithelium No such difference was found in the base regions of the villi The significance of these findings are discussed in relation to current views on the association of epithelial cells and lymphocytes during the development of lymphoid tissues

The kinetics of the intestinal epithelium has been the object of several studies Labelling of proliferating cells with tritiated thymidine and subsequent autoradiographic studies have elucidated the mechanism of cellular renewal in the gastro-intestinal tract It has been established that the epithelial cells originate in the crypts of Lieberkuhn and migrate up the sides of the villi When they reach the tips of the villi the cells are extruded into the lumen of the intestine In most laboratory animals the migration from crypt to villous tip takes 2 to 3 days

The presence of lymphocytes within the intestinal epithelium here called thelio lymphocytes has been described long ago The explanations given for their occurrence at this site have been either that they migrate through the epithelium to the lumen of the intestine or that they form a protective barrier against intestinal bacteria and toxins (13 24) Studies of rodents and man have now shown that the migrating movement of the

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theliolymphocytes takes place in both directions across the basement membrane (18)

Fichtelius (9) studied the kinetics of the theliolymphocytes in germfree and conventional rats Some 2–3 per cent of the theliolymphocytes were shown to synthesize DNA *in situ* and as many as 12 per cent of the theliolymphocytes in the top regions of the villi were less than 3 days old There were no clear cut differences between the germ free and conventional animals in these respects In mice, the labelling patterns of the theliolymphocytes at different times after labelling has been found to be consistent with that of rats, but the labelling indices are considerably lower (7)

During the past few years the association between gut epithelium and lymphoid tissue has been stressed (*1*, *3*, *11*), and some new theories concerning the role of the gut associated lymphoid tissues have been advanced (6, 8) According to one of them the mammalian gut epithelium or specialized parts of it may influence the lymphocytes within the epithelium in a way analogous to the bursa of Fabricius in the chicken (8) The immune apparatus in the chicken is subdivided into two functional systems the thymus and the bursa systems, mediating cellular and humoral immunity respectively (5, 25) Both the ,mus and the bursa of Fabricius are con ..red to be first level lymphoid organs with rapid proliferation of lymphocytes (10) . birds are unique in having a bursa of Fabricius and the delineation of the lymphoid organs referred to above, it seemed of particular interest to study the theliolymphocytes in the chicken

The purpose of this study was to obtain some information on the kinetics of the theliolymphocytes in the ileum of 7 week old chickens, by means of autoradiography and to make a comparison with the kinetics of the peripheral blood lymphocytes

MATERIAL AND METHODS

Animals

Thirty two 7 week old chickens weighing 340 grams (range 300 370 grams) were used They were obtained on the morning of the *21st* day of incubation and raised for the next 2 weeks in heated batteries. Later they were kept in large cages with wire flooring in our animal quarters All were male white Leghorn hybrids of the Babcock *B 300* strain and were taken from the same hatch.

Labelling

Undiluted tritiated *thymidine* ^3H TdR (New England Nuclear Corp, Boston Mass, USA) with a specific activity of 6 7 c/mM and a concentration of 0 036 mg/ml was slowly injected intravenously with a 250 µl Hamilton® syringe fitted with a gauge 22 needle (external diam. 0 30 mm) The dose was adjusted to 0 5 µc per gram body weight All injections were given between noon and 3 p m

Biopsy and Histological Technique

One hour, 48 hours and 6 days after labelling respectively, groups of 8 animals each were killed by the introduction of 10 ml of air into a wing vein Biopsies from the small intestine closely proximal to the ileo coliccaecal junction were taken They were cut along the mesentery pinned on to cork and immersed overnight in carbonate buffered 4 per cent formalin After embedding in paraffin wax the intestinal specimens were sectioned perpendicularly to the muscle layer giving villi cut longitudinally The sections 5 microns thick were attached to gelatine coated slides (23) In order to avoid counting the same cell more than once only every fourth serial section was taken for examination In addition every slide was supplemented with 1 section from unlabelled intestine The slides were deparaffinized in xylene and brought through decreasing grades of alcohol to distilled water A fourth group of 8 chickens was bled 1 hour 48 hours and 6 days after labelling The blood smears were fixed in absolute methanol for 10 minutes

Autoradiographic Technique

The microautoradiographs were produced by the 'dipping' technique described by *Kopriva* & *Leblond* (16) with slight modifications partly according to *Rogers* (23) The undiluted NTB 2 emulsion (Eastman Kodak Comp Rochester NY USA) was melted in a water bath at + 42°C for 45 minutes before starting the procedure The slide was then immersed in the dipping jar and held in the vertical position in the emulsion for 10 seconds after which it was slowly withdrawn With the slide still in the vertical position the emulsion was allowed to drain off for 10 seconds back into the dipping jar and then for another 10 seconds on to a moistened filter paper The slides were then allowed to dry in the horizontal

position for 1½ hours The conditions in the darkroom were adjusted to 20–22°C air temperature and 75–80 per cent relative humidity at the start of the dipping No red safelight was used in the darkroom About 60 slides comprised one dipping batch. The exposure was performed for 21 days at + 4°C in a refrigerator with the slides in light proof stainless steel boxes supplied with silica gel The emulsion was then developed in Kodak D 19B for 2 minutes and fixed in Kodak F 24 for 10 minutes and finally rinsed in distilled water for 20 minutes The temperature of the processing liquids was maintained at + 16°C After rinsing the slides were allowed to dry under a fan at room temperature Light fogged control slides showed no negative chemography

The slides with ileum sections were stained with methyl green and pyronine according to *Kurnick* (17) The blood smears were stained with May – Grunwald and Giemsa stains

Microscopy and Evaluation of Micro autoradiographs

The examination of every slide, using an oil immersion objective giving 1000 × magnification, started with the section from unlabelled control intestine, where the grains over 200 mononuclear infiltrating cells in the epithelium were counted The criterion of a truly labelled cell could then be established on this slide by adding 1 grain to the number of grains over the heaviest labelled mononuclear cell Thus in this experiment a cell with 3 grains was considered as labelled

In sections from labelled intestine the epithelium of longitudinally sectioned villi was subdivided into three regions, viz. top, middle and base The extreme tips of the villi were excluded from the top region, and the middle region was not examined In the top and the base regions, respectively, 200 mononuclear cells, lymphocytes and globule leucocytes were scored and the number of grains over their nuclei was recorded Further, the actual position of the cell vis-à vis the row of epithelial cell nuclei was noted and the levels of the epithelium designated apical intermediate and basal (2 4) The 200 mononuclear cells were scored on 3 different sections on the same slide, about one third on each section All the counting was performed by a specially trained technician

Statistical Methods

Significance analyses of differences were performed with Student s t test

RESULTS

The results from examination of the micro autoradiographs of villous epithelium from ileum of 7-week-old chickens are presented in Table 1 In the epithelium of the top regions of the villi 1 4 per cent of the theliolymphocytes were labelled 1 hour after ³H-TdR administration This means that this 1 4 per cent were in DNA synthesis at the time of labelling During the next 47 hours the proportion of labelled theliolymphocytes increased to 4 0 per cent (P < 001). Four days later, i e 6 days after administration of ³H-TdR, only 3 0 per cent of the theliolymphocytes were labelled, but this decrease is not significant (P < 20) The proportion of labelled theliolymphocytes within the epithelium of the base region of the villi was about the same at corresponding times, thus, 1 hour after intravenous administration of ³H-TdR 1 9 per cent of the theliolymphocytes were labelled The labelling index, i e the percentage of labelled theliolymphocytes increased up to 5 0 per cent 48 hours after labelling (P < 05) and decreased again over the next 4 days to 2 8 per cent (P < 05) On comparing the labelling indices of the theliolymphocytes in the top and base regions of the villi, no significant differences were found at the various times after labelling A statistical analysis of the difference between the labelling index in the base region at 1 hour and in the top region at 48 hours showed this to be significant (P < 005)

Table 2 shows the distribution of labelled theliolymphocytes within the epithelium of the two different regions, top and base One hour after labelling the ratio between the labelling indices of the apical zone and the

TABLE 1 *Labelling Index of the Theliolymphocytes in Top and Base Regions of the Villi at Different Times after Labelling*

Time after ³H TdR	No of animals	Labelling index* of the top region	Labelling index* of the base region
1 hour	8	1 4±0 3	1 9±0 5
48 hours	8	4 0±0 3	5 0±0 7
6 days	8	3 0±0 5	2 8±0 5

* Mean ± standard error of the mean

TABLE 2 *Distribution of Labelled Theliolymphocytes Within the Various Zones of the Epithelium at Different Times after Labelling*

Time after ^{3}H TdR	No of animals	Top region: *a* Labelling index* of apical zone	Top region: *b* Labelling index* of basal and intermediate zones	Top region: Ratio a/b	Base region: *a* Labelling index* of apical zone	Base region: *b* Labelling index* of basal and intermediate zones	Base region: Ratio a/b
1 hour	8	1 9±0 8	1 3±0 2	1 5	2 3±1 3	1 7±0 3	1 4
48 hours	8	7 7±1 8	3 0±0 4	2 5	5 5±1 4	4 8±1 0	1 1
6 days	8	4 2±1 8	2 6±0 4	1 6	2 0±1 1	3 1±0 6	0 6

* Mean ± standard error of the mean

basal and intermediate zones was well above 1 0 and about the same in both top and base regions In the base region the ratio decreased with time and 6 days after labelling it was 0 6 The top region, on the other hand, displayed a different picture At 48 hours after labelling the labelling index of the apical zone was significantly higher than that of the basal and intermediate zones ($P < 05$) Further, in this region the ratio referred to above remained the whole time on a level higher than 1 0

The labelling indices of the lymphocytes in the peripheral blood 1 hour, 48 hours and 6 days after labelling are given in Table 3 One hour after labelling, 1 2 per cent of the blood lymphocytes, exclusively large lymphocytes, were labelled Later, 48 hours after [illegible] 2 6 per cent, mostly small lympho[illegible], were labelled This increase is significant ($P < 001$) Six days after labelling, only 1 3 per cent of the blood lymphocytes were still labelled, which meant a significant decrease during the previous 4 days ($P < 005$) When the labelling indices of the lymphocytes in the intestinal epithelium and in the peripheral blood were compared, no difference was found in the values 1 hour after labelling, but at both 48 hours and 6 days after the administration of ^{3}H-TdR there were significantly higher proportions of labelled lymphocytes in the intestinal epithelium ($P < 005$(top), $P < 02$(base) and $P < 02$(top), $P < 025$(base), respectively)

The mean grain counts of the labelled epithelial columnar cells and labelled theliolymphocytes at different times after labelling are given in Table 4 In comparison with the corresponding values for the theliolymphocytes in the same sections, the true epithelial cells showed significantly higher mean grain counts The mean grain count of the theliolymphocytes 1 hour after labelling was significantly higher than at 48 hours ($P < 02$) No difference was observed between the theliolymphocyte mean grain counts 48 hours and 6 days after labelling

DISCUSSION

In studies of the kinetics of the theliolymphocytes it is important to consider the process of intestinal epithelial cell renewal In most species, including the chicken the cell transit time from the proliferation zone in the crypt to the tip of the villus is 2–3 days (14) The

TABLE 3 *Labelling Index of the Lymphocytes in Blood Smears at Different Times after Labelling*

Time after ^{3}H TdR	No of animals	Labelling index* of lymphocytes	No of scored lymphocytes per animal
1 hour	8	1 2±0 1	400
48 hours	8	2 6±0 2	200
6 days	8	1 3±0 3	200

* Mean ± standard error of the mean

TABLE 4 *Mean Grain Counts over Labelled Theliolymphocytes and Epithelial Cells at Different Times after Labelling*

Time after ^{3}H TdR	No of animals	Mean grain counts* of theliolymphocytes	Mean grain counts* of epithelial cells	P
1 hour	8	13 9±2 2	19 9±0 9	< 05
48 hours	8	6 8±0 4	11 3±0 6	< 001
6 days	8	5 8±0 5	7 6±0 4	< 02

* Mean ± standard error of the mean.

lymphocytes intermingling with the epithelial cells probably join these cells during their migration up the villus This will mean that the lymphocytes at the villous tip either are extruded to the intestinal lumen or migrate back to the lamina propria

The labelling index 1 hour after labelling represents the proportion of theliolymphocytes synthesizing DNA *in situ* There was no significant difference between the top and base regions in this respect The increase of the labelling index between 1 and 48 hours can be explained either by an immigration of labelled cells from the lamina propria or, less likely, by mitosis of the lymphocytes within the epithelium The mean grain count 48 hours after labelling was about half of that at 1 hour, indicating the involvement of mitosis On the other hand, there was no difference between the mean grain counts 48 hours and 6 days after labelling During this 4 day interval, however, the epithelium has been renewed and the whole theliolymphocyte population represents immigrants Therefore, the high labelling index 48 hours after labelling probably expresses the effect of immigration of labelled lymphocytes from the lamina propria

While the epithelial columnar cells slide from the proliferating crypt to the shedding tip of the villus they differentiate to mature absorption cells (22) In accordance with this, most of the absorption takes place in the top region of the villus (15) This will imply that the micro-environment for the intermingling theliolymphocytes is different in different regions of the villus epithelium In the top region there was an accumulation of labelled theliolymphocytes in the apical zone The mechanism behind this observation is obscure One possibility is that the young theliolymphocytes actively migrate to places where they can meet antigens Another possibility, less likely however, would be the effect of a transepithelial migration of the lymphocytes

In the micro autoradiographs from the ileum 48 hours and 6 days after labelling there was a significantly higher proportion of labelled lymphocytes than in the corresponding blood smears This indicates an accumulation of newly proliferated lymphocytes within the intestinal epithelium This observation is in agreement with previous reports (7, 9) The accumulation of young lymphocytes also seems to be valid for other epithelia, e g epidermis (12) In this context it is interesting to look for the relevance of other associations between lymphocytes and epithelium During the development of the bursa of Fabricius and the thymus, both considered to be first level lymphoid organs, there is a close relationship between the epithelial primordia and the proliferating stem cells derived from the blood stream (19, 20, 21) The presence of the bursa of Fabricius in birds does not exclude a bursal type function of the intestinal epithelium, which in some way may influence the accumulating newly proliferated theliolymphocytes

The work was supported by grants from the *Medical Faculty of the University of Uppsala* and the *Swedish Medical Research Council*

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Acta path microbiol scand Section A 80, 97–108, 1972

ON THE HISTOPATHOLOGY OF LIVER AND LIVER TUMOURS IN THORIUM-DIOXIDE PATIENTS

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Among 5819 autopsies carried out over the years 1966 to 1969, 6 cases were found having thorotrast deposits in the liver One case presented non specific reactive hepatitis, and 5 cases had cirrhosis The cirrhoses were of the irregular type, in 4 cases with recent and long lasting confluent necroses, in all 5 cases with extensive passive septa The morphogenesis of the thorotrast cirrhosis is discussed on the basis of the histological changes Four of the patients had malignant tumours which are likely to be induced by thorotrast deposits in the liver mesothelioma angiosarcoma, hepatoma and cholangiohepatocarcinoma Angiosarcomas develop relatively frequently in the liver in thorotrast patients, and in the majority of cases

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administration

In Denmark, thorotrast was used during the years 1932 to 1947 Except in a few cases, the contrast medium was used exclusively for cerebral angiography (3) Upwards of 1000 patients injected with thorotrast, have been recorded In 1966 about 50 per cent of these patients had died (3) Because the majority of the patients who are still alive, are elderly persons and because the late effects of thorotrast injections are likely to become manifest an appreciable number of these patients must be expected to be autopsied at the Danish institutes of pathology in the near future

It is of particular importance to study these cases thoroughly, because the autopsies concerned will present patho-anatomical changes which it will only be possible to study for a limited number of years To this should be added that, during recent years, the interest in and knowledge of liver pathology have increased considerably Among other things this is due to the fact that it has become possible, by means of repeated percutaneous liver biopsies, to follow up the histological changes occuring during various stages of a number of liver diseases

Experience gained from such investigations formed the basis of the present studies of the liver pathology in a limited number of patients, in whom the late effects of the thorotrast varied greatly Comments will also be made on the induced tumours and the indications for percutaneous liver biopsies in thorotrast patients

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TABLE 1 *Liver Pathology in 6 Thorotrast Patients from 5819 Consecutive Autopsies*

No	Age at death	Sex	Year of injection	Dose cm³	Years with thorotrast	Year of autopsy	Microscopy No	Cause of death	Histopathologic liver diagnosis
1	44	♂	1938	20	29	1966	LB 1906/66 S 5173/66	Mesothelioma of peritoneum	*Mesothelioma of peritoneum* Non-specific reactive hepatitis and thorotrast
2	42	♂	1938	50	29	1967	S 1853/67	Hepatic coma	Angiosarcoma of liver Liver cirrhosis (irregular) with thorotrast and extensive confluent necroses
3	65	♂	1942	20	25	1967	LB 2905/67 S 4339/67	Haemorrhage from oesophageal varices	Liver cirrhosis (irregular) with thorotrast and passive septa
4	74	♀	1944	?	24	1968	S 4929/68	Liver cirrhosis with *portal vein trombosis*	Liver cirrhosis (irregular) with *thorotrast and confluent* necroses and cholestasis
5	50	♂	1944	30	25	1969	1282/69 S 1350/69	Hepatoma with metastases	Hepatoma Liver cirrhosis (irregular) with thorotrast and extensive confluent necroses
6	68	♂	1944	28	25	1969	S 4519/69	Cholangiohe-patocarcinoma and abscessforming cholangitis	Cholangiohepatocarcinoma Liver cirrhosis (irregular) with thorotrast and extensive confluent necroses and cholestasis Abscess-*forming* cholangitis

MATERIAL AND METHODS

Over the years 1966 to 1969, 5819 autopsies were carried out at the Institute of Pathology, Kommunehospitalet, Copenhagen Throughout this four year period, routine microscopy of liver tissue was made from all autopsies Among the 5819 consecutive autopsies, 6 cases were found with thorotrast deposits in the liver tissue

Only routine histological examinations were made, and no special efforts were made to trace thorotrast and consequently, the possibility cannot be excluded that occasionally thorotrast deposits have been overlooked

Routine staining with haematoxylin and eosin, as well as van Gieson Hansen staining, was made in some cases supplemented by staining for reticulin (4), iron (7) and pyronine (Unna Pappenheim) Autoradographic studies were not performed

In addition to the autopsy microscopy, percutaneous liver biopsies were available in 3 of the 6 patients (Nos 1, 3 and 5), in all 3 cases obtained within the same year as the autopsy

RESULTS

Table 1 presents a number of data on the patients It appears that the patients had received the intra arterial thorotrast injection between 24 and 29 years before death The dosage was known in 5 of the patients and ranged between 20 and 50 ml, which must be considered high doses

The causes of death are stated for all the 6 patients, in that the assessment is based both on clinical records and on the autopsy records It will be seen that all the patients died from diseases which must be regarded as late effects of the thorotrast injection In 5 of the 6 patients cirrhosis had developed In 4 of the 6 patients malignant tumours were found, i e mesothelioma angiosarcoma, hepatoma and cholangiohepatocarcinoma

Macroscopical Findings at Autopsy

Patient No 1

The liver measured 24×16×8 cm The lower surface was covered by whitish tumour tissue thickness about 1 cm The cut surface presented normal lobular architecture with signs of chronic stasis On the parts of the intestine and stomach covered by the peritoneum numerous small whitish tumour processes were found No spleen could be found, its normal position was occupied by tumour tissue and a calcified area, measuring about 2 cm in diameter

Patient No 2

The liver was enlarged, measuring 32×24×10 cm The surface was irregularly nodular, mottled, dominated by prominent tumour masses In some areas, coarse granular liver tissue was observed The cut surface presented small areas of cirrhotic liver tissue, but everywhere else it was dominated by tumour tissue with abundant bleeding and large cystic blood filled cavities as well as yellowish necroses No metastases were revealed

Patient No 3

The liver measured 21×15×6 cm The surface was coarsely granular, grey yellowish Similar coarse granulation was seen on the cut surface No tumour suspect areas were observed In the lower third of the oesophagus pronounced varices were found, and in the vicinity of these varices there were two small ulcerations of the mucous membrane

The stomach contained about 200 ml of coagulated blood, and the intestine contained fresh blood extending as far as to the rectum.

Patient No 4

The liver was diminished in size, measuring 20×11×6 cm. The surface was very irregular nodular and on the cut surface the lobular architecture had disappeared completely and there were coarse irregular nodules The tissue was yellowish and of an extremely increased consistency There was thrombosis in the portal vein and the abdominal cavity contained 1 5 litres of ascites fluid

Patient No 5

The liver measured 24×18×7 cm The surface was coarsely nodular A 10×12 cm area, marginally on the right lobe, was grey reddish and on sections corresponding to this area there was pale, grey reddish, relatively firm tumour tissue, the outline of which was

poorly demarcated from the remaining liver parenchyma Apart from this, no focal changes were found The consistency of the liver tissue was somewhat firmer than normal, and it was difficult to evaluate the architecture Diffuse carcinosis was found in the mesenterium and the omentum, as well as tumour elements, up to 0 5 cm in size, around the gall bladder There were 3 litres of cloudy, greyish ascites fluid

Patient No 6

The liver was small, measuring 15×15×8 cm The surface was partially covered by pus originating from a subphrenic abscess The cut surface was rather coarsely granular, greenish-black, of a firm consistency, and in the porta hepatis, extending about 5 cm into the liver parenchyma, large whitish firm tumour masses were observed, obstructing the bile ducts

Microscopic Examination of Liver Tissue

In patient No 1 only very slight changes were found in the liver itself (Fig 1) The lobular architecture was normal The portal tracts were not enlarged, they were without bile duct proliferation, but in some areas slight infiltration of lymphocytes and histio s was observed There were moderate ntities of thorotrast in the connective

In the parenchyma a few small focal liver-cell necroses with moderate Kupffer cell proliferation were seen The necroses were not predominantly zonal, and the shape and size of the liver cells and the liver-cell nuclei did not vary greatly (Fig 2) There was no steatosis A few Kupffer cells containing thorotrast were found (Fig 3), but no thorotrast was observed in the liver cells or in relation to the liver cell necroses

The liver tissue specimens from the remaining 5 patients presented cirrhosis, defined as nodular regeneration and fibrosis In all the cases the cirrhosis was of the irregular type with nodules, almost all of which were considerably larger than normal lobules (Fig 4), and presenting moderately or markedly increased connective tissue (Fig 5)

It was a characteristic feature that large quantities of thorotrast were found in the connective tissue (Fig 5), mainly free, and to a lesser degree in macrophages Generally, there was only slight bile duct proliferation and very slight infiltration of lymphocytes, plasma cells and histiocytes

In 4 cases (patients Nos 2, 4, 5 and 6), dispersed, small focal liver cell necroses were found in addition to typical confluent necroses (Figs 6 and 7), i e, necroses affecting substantial groups of adjacent liver cells (6) The confluent necroses were often large, and in some areas there were also massive necroses comprising portions which were of the same size as the lobules, or larger

Many of the confluent necroses were of recent origin, with distinct remnants of the original liver cells (Figs 6 and 7), whilst others were more or less fibrotic and formed so-called passive septa (Figs 8 and 9), i e, connective tissue septa, arising in areas with former confluent necroses, with only few inflammatory cells (Fig 10) The shape and size of the existing liver cells did not vary greatly

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Fig 1 Non specific reactive hepatitis In the centre is seen a portal tract with slight inflammatory cell infiltration and at the lower left a focal liver cell necrosis × 100 (Patient No 1

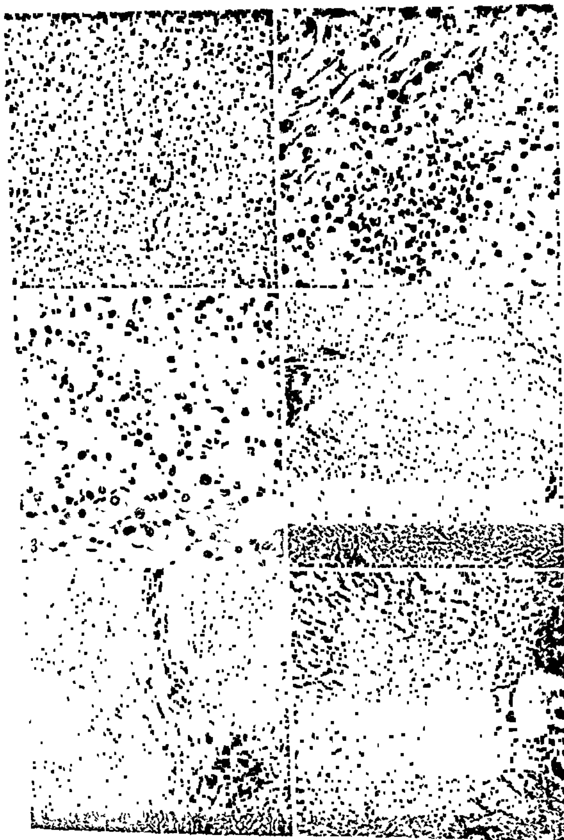
Fig 2 Higher magnification of focal liver-cell necrosis from Fig 1 Note the slight variation in the shape and size of the surrounding liver cells × 250

Fig 3 Slight proliferation of Kupffer cells Thorotrast material is seen mainly in the Kupffer cells in the central part of the picture × 250 (Patient No 1).

Fig 4 Part of large nodule with passive septum in the upper part of the picture × 100 (Patient No 3)

Fig 5 Area with pronounced fibrosis and large amounts of thorotrast aggregates in the connective tissue At the lower left part of a confluent recent necrosis × 100 (Patient No 2

Fig 6 Recent confluent necrosis × 100 (Patient No 2)


3


12

In one case (patient No 3), no confluent necroses of recent origin were observed, only passive septa Apart from this finding, this case differed from the remaining cases in that so called active septa could be seen around the original portal tracts (6) Hereby is understood irregular, more or less pronounced connective tissue proliferation, extending from the portal tracts, with pronounced infiltration of inflammatory cells Contrary to those of the passive septa, the outlines of the active septa were blurred In the same areas, the bile duct proliferation was more pronounced than elsewhere

Diffuse, fairly pronounced cholestasis was observed in two patients (Nos 4 and 6) In Nos 4 and 5 there was severe (Fig 11), and in No 6 slight adenomatous liver cell proliferation in the same areas Furthermore, No 6 had abscess forming cholangitis

Steatosis or haemosiderosis was not demonstrated

Similar to the findings in the first case, only small quantities of thorotrast were found in the liver parenchyma itself The deposits were found mainly in the Kupffer cells and, supposedly, in a few liver cells (Fig 12)

The histological features of the biopsy specimens and of the tissue sections obtained by autopsy in the three patients (Nos 1, 3 and 5) showed complete concurrence

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Fig 7 Part of confluent necrosis At the upper right part of a portal tract Higher magnification of Fig 6 × 250 (Patient No 2)

Fig 8 Passive septum with a few intact liver cells Note the slight infiltration of inflammatory cells × 100 (Patient No 4)

Fig 9 Same as Fig 8

Fig 10 Higher magnification of passive septum In the upper central part of the picture, portions of an original portal tract can be recognized × 250 (Patient No 4)

tory cells × 100 (Patient No 5)

Fig 12 High magnification of thorotrast granules possibly in liver cells × 450 (Patient No 2)

Microscopical Examination of Tumours

Patient No 1 (*mesothelioma*)

The pattern of the tumour tissue varied greatly from one area to another (Figs 13–15) Solid areas were seen, in which the tumour cells were of the spindle cell type (Fig 15), whereas in other parts there were cords of cells, clefts or lumina covered with cuboidal tumour epithelium (Figs 13 and 14)

The histological picture was characteristic of a mesothelioma No thorotrast was found in the tumour tissue, but abundant quantities in the liver with considerable accumulation in the thickened fibrous capsule and in the liver parenchyma beneath the capsule

Patient No 2 (*angiosarcoma*)

The tumour tissue consisted of large polymorphic hyperchromatic tumour cells (Figs 16 and 17), often in whorled blood filled patterns or around large blood filled cavities There were many tumour necroses and infiltration of tumour tissue in the liver parenchyma Dense thorotrast deposits were observed surrounding the tumour tissue and the tumour necroses There was free thorotrast in the tumour tissue and supposedly some tumour cells contained thorotrast granules (Fig 18)

Patient No 5 (*hepatoma*)

The appearance of the tumour tissue in different areas varied greatly, presenting preserved areas and areas with necrotic foci of varying size The tumour was rather highly differentiated (Figs 19–21) and poorly demarcated (Fig 20) There were areas with solid structure and areas with trabecular (Fig 21) or adenomatous structure It is characteristic that larger amounts of thorotrast were found in the connective tissue adjacent to the tumour (Fig 19) Possibly, a few tumour cells contained thorotrast granules

Patient No 6 (*cholangiohepatocarcinoma*)

There were large areas with rather low differentiated hepatoma tissue (Fig 22),

often surrounded by thorotrast deposits, or thorotrast being present in connective tissue in the tumour. Furthermore, areas with typical cholangiocarcinoma presenting irregular clefts and lumina were seen, lined with cuboidal or columnar tumour epithelium (Figs. 23 and 24). There were extensive necroses.

Occasionally hepatoma cells were found, apparently containing a few granules of thorotrast.

DISCUSSION

In a number of countries, a register of thorotrast patients has been set op. This renders it possible to collect a series of data, mainly on the late effects of thorotrast. Hence, the Register at the Finsen Institute in Copenhagen receives information from all pathological institutes in Denmark and, generally, both autopsy records, specimens and microscopy reports are submitted. Consequently, the six patients, of whom the liver pathology is described in the present paper, are also included in the Danish Register at the Finsen Institute.

Hepatic Changes following Administration of Thorotrast

Numerous studies have been carried out [illegible] thorotrast deposits following intravasal application. A great many experimental studies have been published, and it has also been attempted, through several clinical studies to clarify the patho-physiological and patho-anatomical conditions following different doses of thorotrast and after varying periods of time. Only brief mention will be made of the most important results relating to our investigation, in particular the findings described below concerning development of necroses and cirrhosis in thorotrast livers.

The uptake of thorotrast occurs in the reticuloendothelial system and, after a short period, the major amounts are concentrated in the liver and spleen. The most important changes occurring in the human liver, on which there seems to be a consensus of opinion, have been summarized e.g. by *Tessmer & Chang* (10). Initially there is uniform distribution of thorotrast in the Kupffer cells, small amounts are found in the liver cells or free intracellularly. Subsequently, acute degenerative changes occur in the liver cells with diffuse as well as focal degeneration and necrosis. Later there will be formation of aggregates of thorotrast granules, both free and phagocytized with increased connective tissue, primarily in the portal tracts but also in the hepatic lobules. In certain cases only slight response to the thorotrast is seen. Finally, there is evidence that a redistribution of thorotrast occurs, often with larger accumulations of free material in the fibrous tissue. The mechanisms governing this redistribution are still being discussed, but it is of importance that the total amount of thorotrast in the liver does not seem to change significantly (5).

From our histological studies of the liver tissue specimens obtained from the 6 patients comprised by the present investigation in whom thorotrast was administered from 24 to 29 years previously, some of these features were observed.

Patient No. 1 belongs to the cases showing slight response to thorotrast. The histological picture corresponds to the findings normally

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Fig. 13. Mesothelioma with clefts and lumina lined with epithelial tumour cells. × 100. (Patient No. 1)

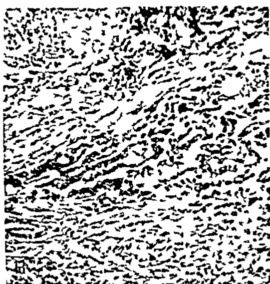
Fig. 14. Higher magnification of Fig. 13. × 250.

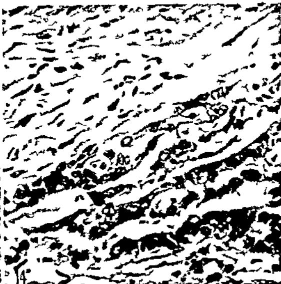
Fig. 15. Mesothelioma, same tumour as that shown in Figs. 13 and 14. Portion with sarcomatous pattern. × 100. (Patient No. 1)

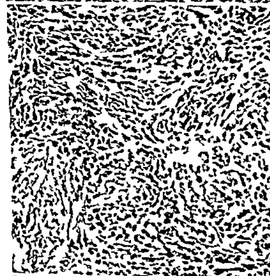
Fig. 16. Angiosarcoma with pronounced cellular and nuclear polymorphism. × 100. (Patient No. 2)

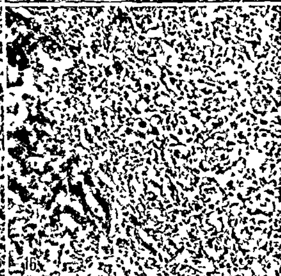
Fig. 17. Higher magnification of angiosarcoma with vascular clefts with red blood cells, particularly to the left. × 250. (Patient No. 2)

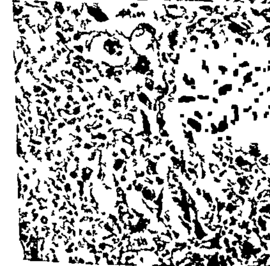
Fig. 18. High magnification of thorotrast granules, lying freely and possibly in fusiform tumour cells from an angiosarcoma. × 450. (Patient No. 2)

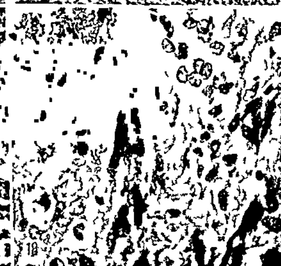


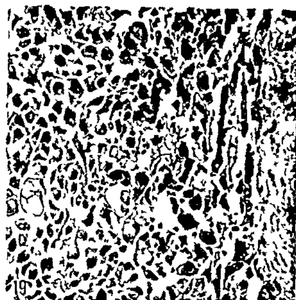

14

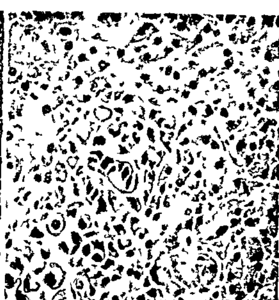


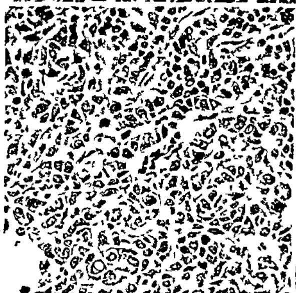

16

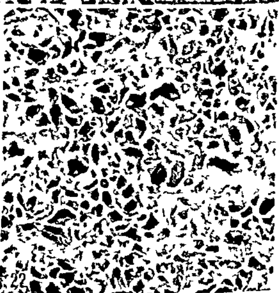



18


19






22





made in so-called non specific reactive hepatitis (9)

It is worth noting that the amounts of thorotrast found in the areas of the parenchyma presenting small focal necroses were not larger than those found in areas without necroses It is not possible on the basis of the data available at present, to express any definite opinion as to whether the slight changes in the hepatic tissue are produced by existing tumour tissue or are related to the thorotrast However, in spite of the fact that a period of 29 years had elapsed since the administration of thorotrast it must be justifiable to state that the liver changes observed in this patient are not of the same quantity and supposedly, also not of the same quality as those found in the remaining 5 patients Although dispersed small focal liver-cell necroses were also found in the other patients the histological picture in these cases was completely dominated by confluent necroses and their sequels Following a review of the specimens it does not seem probable that the small focal necroses are connected in any way with the development of cirrhosis

The five cirrhoses are all of the irregular type (8), and in four of the cases both recent and long lasting confluent necroses are seen whereas, in the fifth case, only long lasting necroses were found In many areas the connective tissue which is formed in relation to these confluent necroses is undoubtedly the septa, so-called passive septa, which surround the generally large nodules

Cirrhosis occuring after acute viral hepatitis in some cases may develop following confluent necrosis with the formation of passive septa or it may develop after a stage of chronic aggressive hepatitis with active septa (6) Although the present study comprises a limited number of patients only, it is reasonable to suppose that the morphogenesis of cirrhosis in thorotrast patients follows parallel patterns. Most likely the development of cirrhosis after administration of thorotrast takes place mainly via confluent necroses and passive septa The confluent necroses were found in 4 of the 5 patients with cirrhosis passive septa were observed in all of them

It does not appear from the histological studies how the thorotrast acts in the formation of the confluent necroses or the passive septa Larger amounts of thorotrast were not observed in areas with confluent necroses than in the remaining portions of the hepatic parenchyma Conversely there were large quantities of contrast medium in the finished septa, consisting of collagen rich poorly vascularized connective tissue

The present investigation does not provide any clear solution to the problems connected with the redistribution of thorotrast, but the fact that only very few or generally no thorotrast granules are found in the recent confluent necroses whereas large amounts are found in the fully developed septa supports the theory that transport of thorotrast occurs Furthermore this is supported by the fact that also after 24 to 29 years thorotrast can be demonstrated lying freely between liver cells and supposedly also in the liver cells, without any liver-cell necroses being observed in the areas concerned

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Fig 19 Rather highly differentiated malignant hepatoma with tumour cells arranged irregularly In the connective tissue at the upper right thorotrast deposits are seen. × 250 (Patient No 5)

Fig 20 The same tumour as that shown in Fig 19 Invasive growth is seen in areas with normally differentiated liver cells × 250

Fig 21 The same tumour as that shown in Figs 19 and 20 The tumour tissue here has a more trabecular and adenomatous structure × 250

Fig 22 Fairly low differentiated hepatoma from cholangiohepatocarcinoma × 250 (Patient No 6)

Fig 23 Another area of the same tumour as that shown in Fig 22 showing cholangiocarcinoma × 100

Fig 24 Higher magnification of cholangiocarcinoma from areas adjacent to that shown in Fig 23 × 250

Malignant Tumours following Administration of Thorotrast

The application of thorotrast may result in a number of malignant diseases. Numerically, leukaemia and various liver tumours are the predominant disorders.

To produce satisfactory evidence for a relationship between thorotrast and the malignant tumours, as emphasized *e. g.* by *Dahlgren* (1), the following criteria must be fulfilled:

1) thorotrast deposits should be found in the immediate vicinity of the primary tumour,
2) the latent period should be sufficiently long,
3) the amount of thorotrast and, consequently, the radiation dose should be sufficiently high.

The 4 cases with malignant tumours described in the present paper fulfil these criteria. Thorotrast induced mesotheliomas have been described but on rare occasions only (see *e. g. Dahlgren* (2)). It must be considered probable that the mesothelioma described in the present paper was induced by the thorotrast in the liver and in its fibrous capsule.

Angiosarcomas, hepatomas and cholangiocarcinomas are well known occurences in thorotrast patients. Among the malignant liver tumours induced by thorotrast the angiosarcomas are by far the most frequent. Angiosarcomas in the liver which are not *induced by thorotrast are rare.*

Thorotrast patients are often referred to hospitals because of impaired liver function and consequently liver biopsy will often be contemplated. In the case of angiosarcoma described in the present paper (patient No. 2), the tumour tissue contained large blood filled cavities which is a characteristic

and therefore angiography should always be made before liver biopsy in patients in whom previous administration of thorotrast is known or suspected.

The authors gratefully acknowledge the information regarding thorotrast dosage in some of the patients submitted by Professor *M. Faber*, the Finsen Institute, Copenhagen.

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Acta path. microbiol scand Section A 80, 109–114, 1972

IMPAIRMENT OF THE LYMPHOCYTE RESPONSE TO PHYTOHAEMAGGLUTININ IN CHICKENS WITH MAREK'S DISEASE

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and
United States Department of Agriculture, Agricultural Research Service, Regional Poultry Research Laboratory, East Lansing, Michigan, U S A

Spleen lymphoid cells obtained from White Leghorn chickens with Marek's disease exhibited a significantly impaired proliferative response to the mitogen phytohaemagglutinin (PHA) as measured by the incorporation of tritiated thymidine While chickens infected by the intraperitoneal inoculation of infectious blood on the day of hatching still were able to mount a small PHA response, their contact infected pen mates completely failed to respond Lymphoid tumour cells from the gonads of inoculated chickens with Marek's disease were not stimulated by PHA Rather a suppressive effect of PHA on these cells was noted

Marek's disease (MD), is a contagious lymphoproliferative disease belonging to the avian leucosis complex The histopathological lesions include lymphoid cell infiltrates in the nerves and lymphoid tumours in the gonads and other visceral organs and in the skin (19) The aetiologic agent is a highly cell-associated Group B herpesvirus (5, 6, 14 20, 21) MD specific antigens can be demonstrated in a number of organs of infected animals but little or no MD antigen or virus particles can be detected in peripheral blood and spleen lymphocytes or lymphoid tumour cells (1, 10, 16) Because even small numbers of such cells transmit the disease, they still contain viral genomes (22)

A number of viral infections (8, 12, 15) inhibit the *in vitro* proliferative response of lymphocytes to the mitogen phytohaemagglutinin (PHA) This suggested that the presence of masked MD virus in the lymphocytes could possibly inhibit their response to PHA In the present investigation the response of spleen lymphoid cells from chickens with MD to PHA was therefore investigated

Human lymphoid cells in Hodgkins disease and lymphatic leukaemias (8, 12, 15) have previously been reported to lack the ability to respond to PHA The response of gonadal lymphoid tumour cells in Marek's disease was therefore examined to test whether they behaved like normal lymphocytes or were unresponsive to PHA

Received 28 vi 71

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Thorotrast patients are often referred to hospitals because of impaired liver function and consequently liver biopsy will often be contemplated In the case of angiosarcoma described in the present paper (patient No 2), the tumour tissue contained large blood filled cavities, which is a characteristic finding This involves a considerable risk of fatal bleeding in connection with liver biopsy and therefore, angiography should always be made before liver biopsy in patients in whom previous administration of thorotrast is known or suspected

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Spleen lymphoid cells obtained from White Leghorn chickens with Marek's disease exhibited a significantly impaired proliferative response to the mitogen phytohaemagglutinin (PHA) as measured by the incorporation of tritiated thymidine While chickens infected by the intraperitoneal inoculation of infectious blood on the day of hatching still were able to mount a small PHA response, their contact infected pen mates completely failed to respond Lymphoid tumour cells from the gonads of inoculated chickens with Marek's disease were not stimulated by PHA Rather, a suppressive effect of PHA on these cells was noted

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MATERIALS AND METHODS

Chickens White Leghorn chickens of the inbred line 7 from the U S Department of Agriculture Regional Poultry Research Laboratory in East Lansing, Michigan were used This line is highly susceptible to Marek s disease (MD) but resistant to common strains of lymphoid leucosis viruses (7)

Marek s disease (MD) The JM strain of MD isolated by *Sevoian et al* (18) was used The strain has been maintained by serial passage in chickens at 4 6 week intervals with heparinized whole blood or tumour cell suspensions No change in potency or pathological characteristics has been noted with passage

Experimental infection Chickens were inoculated on the day of hatching with 0 2 ml infected heparinized whole blood intra abdominally Birds referred to as contact infected ' were housed in the same Horsfall Bauer isolators as the inoculated birds and thus infected by these Control birds were held in separate isolators

Lymphoid cell cultures The chickens were sacrified at indicated age by exsanguination from the heart Lymphoid spleen and tumour cell suspensions were prepared and cultured as previously described (3) The culture time was 72 hours at 39° C

Phytohaemagglutinin (PHA) Lyophilized PHA (Burroughs Wellcome and Co , London, England) was reconstituted with 5 ml Dulbecco phosphate buffered saline per vial and added to the cultures in indicated volumes at the initiation of the cultures

Assay of the DNA synthesis At the end of the culture period 1 μCi H^3 methyl thymidine H^3 TdR spec act 6 7 Ci/mM (New England Nuclear Boston Mass U S A) was added to each culture for a 4 hour period The radioactivity in was determined by liquid scintillation counting as previously described (3)

Cytological and histological procedures Cell counts on spleen and tumour cell suspensions were carried out with Natt Herrick s stain (13) From the cell counts the total number of cells obtained per spleen were calculated Biops es of the vagal and sciatic nerves and the brachial plexus of the birds were formalin fixed embedded sectioned and stained with haematoxylin eosin

RESULTS

The inoculated and contact infected birds exhibited at 28 days and 24 days of age respectively, the overt signs of Marek s disease, *i e* beginning paralysis of wings and legs and impaired balance Contact infected animals were in a less advanced stage of the disease than inoculated birds The animals were not emaciated Microscopical examination of histological sections of the nerves showed extensive diffuse or focal infiltration of pleomorphic lymphocytes in 6/8 animals infected by inoculation of infectious blood In the remaining 2 animals in this group as well as in the contact infected birds and healthy controls significant histopathological nerve lesions were not detected

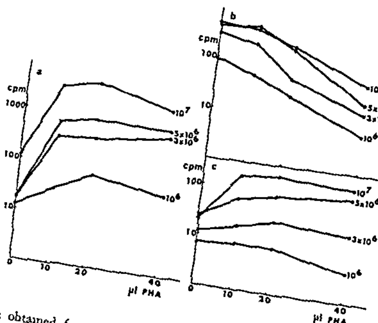
Spleen sizes Table 1 demonstrates the number of cells obtained from the spleens of one group of 8 inoculated and 8 control chickens and one group of 3 contact infected and 3 control chickens, 28 days and 24 days of age, respectively The number of spleen cells obtained from inoculated animals is significantly higher (t = 3 16, P <0 01 than that obtained from normal chicken spleens The spleens from contact infected birds appear to contain more cells than normal birds of the same age but the sample size does not permit a meaningful statistical analysis

TABLE 1 *The Total Number of Cells per Spleen in Inoculated and Contact Infected Chickens with Marek s Disease and in Healthy Controls Mean Number of Cells X 10⁻⁶ ± Standard Error (S E)*

Treatment	No animals	No cells X 10⁶ per spleen	Probability (P)
Inoculated	8	568 ± 179	<0 01
Control	8	334 + 77	
Contact infected	3	710 715 1055	—
Control	3	530 595 560	

PHA responsiveness Table 2 demonstrates that the PHA response of spleen cells obtained from 28 day old inoculated birds with MD is significantly impaired (P <0 01) as compared to those of healthy controls The results are expressed as percent increase in H^3 TdR incorporation caused by PHA

No difference in the absolute H^3 TdR incorporation in the absence of PHA, of spleen



The effect of varying the concentration of PHA (µl per culture) on the incorporation of H³ methyl-thymidine (counts per minute per culture) by a) normal spleen cells, b) gonadal tumour cells, and c) spleen cells from an inoculated chicken with Marek's disease at different cell concentrations (number cells per culture)

cells obtained from normal and inoculated chickens was noted in the experiment of Table 2 (2098 ± 417 resp 2421 ± 540 counts per minute)

TABLE 2 *The in vitro Response to PHA of Normal Spleen Cells, Spleen Cells from Inoculated Chickens with Marek's Disease and Gonadal Tumour Cells**

Treatment	No animals	Change in DNA synthesis*	Probability
Control	13	1287 ± 646	
Inoculated	12	435 ± 581	<0 01
Tumour cells	6	−65 ± 9	<0 001

* Expressed as per cent change in the H³ methyl thymidine incorporation ± standard error (S E) in cultures with 20 µl PHA compared to cultures without PHA

Table 2 also demonstrates the *in vitro* behaviour of lymphoid tumour cells obtained from the gonads of inoculated chickens with MD While these cells normally are rapidly synthesizing DNA the addition of PHA causes a uniform and highly significant (P <0 001) decrease of the H³-TdR incorporation

Figure 1 demonstrates the *in vitro* PHA response of normal spleen cells, spleen cells from inoculated chickens with MD and lymphoid tumour cells at different cell concentrations and PHA concentrations Spleen cells from inoculated animals responded poorly at low cell concentrations At higher cell concentrations a better response was encountered but it never aproached that of the normal cells PHA uniformly decreased the H³-TdR incorporation of tumour cells at all cell concentrations and PHA concentrations

Table 3 compares the *in vitro* response to

TABLE 3 *The in vitro Response to PHA of Normal Spleen Cells and of Spleen Cells from Contact Infected Chickens with Marek's Disease**

Treatment	No cells per culture	Change in DNA synthesis* in animal no 1	2	3
Control	5 × 10⁶	834 9	1961 9	1228 5
	10 × 10	408 0	45 4	451 2
Contact infected	5 × 10	68 9	66 7	71 9
	10 × 10	99 7	50 5	−49 2

* Expressed as per cent change in the H³ methyl-thymidine incorporation in cultures with 20 µl PHA compared to cultures without PHA

PHA of spleen lymphoid cells *from contact* infected and normal 24 day old *chickens* The H^3-TdR incorporation in non stimulated (no PHA added) spleen cells from contact-infected animals was strikingly decreased *both* at a cell concentration of 5×10^6 *and* 10×10^6 cells per ml (495 and 1372 c p *m*) *compared* to spleen cells obtained *from healthy control* animals (5063 and 4957 c p m, respectively) PHA decreased the DNA synthesis in spleen cells from contact infected animals at a cell concentration of 5×10^6 cells *per ml*, while at 10×10^6 cells per ml a small increase in *H^3-TdR incorporation was noted in 2 of the* infected animals The response of normal *spleen cells* at *the high cell* concentration was *poor while* at 5×10^6 cells per ml a more than *tenfold stimulation* of the *DNA* synthesis was noted

It was noted that the animals that failed to respond to PHA, i e *2/8* inoculated and *3/3* contact infected were identical with those lacking significant histopathological nerve lesions

DISCUSSION

Lymphoid tumour cells obtained from the *gonads* of chickens with Marek s disease *(MD)*, completely failed to react to the mito n phytohaemagglutinin (PHA) Also spleen cells from 28 day old birds inoculated at one y of age with infectious blood exhibited a less than normal proliferative response to PHA while spleen cells from contact infected animals failed to react or reacted very poorly to the mitogen It thus appears that a sub stantial fraction of the small lymphocyte population through infection by the MD agent is made unresponsive to PHA *in vivo* This may indicate that these cells contain or once contained the virus The possibility re mains, however, that the PHA reactive lym phocytes were infected first in tissue culture with virus originating from a few infectious cells

It is noteworthy that the PHA caused a uniform and highly significant dose de pendent inhibition of the H^3 TdR incorpor ation of the tumour cells This was seen even at as low a concentration as 10 µl (approxi *mately 10 µg) PHA* per ml culture medium *This* may indicate that the tumour lymphoid cells interact with the PHA but unlike normal lymphocytes are not stimulated to proliferation by the mitogen

Although *both inoculated* and contact *infected birds* showed impaired *PHA* re *sponses*, the differences in the results with *these* groups merit further discussion Non *stimulated spleen cell cultures* from contact *infected chickens* exhibited a significantly *depressed DNA* synthesis compared with control birds and inoculated birds Therefore the enlarged spleens in the contact infected group may not be a result of an accumulation of tumour cells since these have a high rate of DNA synthesis

Furthermore, a difference in the PHA responsiveness of spleen cells from contact infected and inoculated chickens was ob served In the former at optimal cell con centration, PHA actually decreased the syn thesis of DNA At higher cell concentrations a slight increase was noted which indicates that a few PHA reactive cells still remained In contrast the spleen cells from the ino culated chickens showed a significant albeit still impaired response to PHA Birds ino culated with the MD agent at one day of age begin to shed the agent at approximately 14 days of age (9) At that time the contact in fection of their non inoculated pen mates take place The inoculated birds and their contact infected pen mates may therefore differ because they are at different stages of the disease The route of infection or age at infection may also modify the course of the disease

The observation that the birds least re sponsive to PHA corresponded to those lacking significant histopathological nerve lesions further suggest that the stage of the disease may influence the PHA responsiveness It is possible that the lymphocyte response is preferentially suppressed during the incuba tion period of Marek s disease *However*

further studies are required to secure this conclusion

Several viruses have been associated with impaired lymphocyte responsiveness to PHA (8, 12, 15) The present investigation demonstrates a suppressed PHA response in a disease caused by a tumour virus *Purchase* et *al* (16) showed an impairment of the immune response in chickens with Marek's disease These findings, coupled with our data, suggest that in this disease an impairment of the functions of the lymphocytes take place

Cell mediated immune responses may play an important part in the rejection of virus-induced tumours and in the defense against certain virus infections (2) The role of virus induced suppression of the immune response in the oncogenic action of several viruses have been discussed (8 15) Because the effector cells in cellular immunity appear to belong to the same thymus dependent lymphocyte population as the PHA reactive cells (4, 11, 12) it is possible that the fuctional impairment of these cells in Marek's disease is a factor contributing to the extreme contagiosity, morbidity and mortality in Marek s disease and the development of lymphoid tumours

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The authors are indebted to Dr *B R Burmester* for reviewing the manuscript The skilled technical assistance of Mrs *C Mayne* is gratefully acknowledged

This investigation was supported by grants *USPHS Medical Research* A1 08771-01 and *USPHS Medical Research* (Training Grant) 5-T01 AM-05589-2

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Acta path microbiol scand Section A 80, 115–124, 1972

QUANTITATIVE STUDIES OF THE RENAL CORPUSCLES I: INTRAGLOMERULAR, INTERGLOMERULAR AND INTERFOCAL VARIATION IN THE NORMAL KIDNEY

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A quantitative method based on camera lucida drawing and planimetry was used to evaluate intraglomerular interglomerular and interfocal variation with regard to the following parameters 1) differential number of glomerular nuclei 2) mesangial area in percentage of total area 3) total number of nuclei per 1 000 μ^2 of total area and 4) number of mesangial nuclei per 1 000 μ^2 of mesangial area Study of intraglomerular variation revealed that as far as the above-mentioned parameters are concerned a central part is found to comprise approximately half of the glomerulus where values are relatively constant Histograms based on results obtained in 141 normal glomeruli from the same kidney showed a reasonably good approximation to a normal distribution for all parameters concerned Interfocally a statistically significant difference in nuclear density could be demonstrated but this was not the case as regards differential counts and percentage of mesangial area. Comparison of a tissue specimen with subcapsular glomeruli with a tissue specimen with juxtamedullar glomeruli demonstrated no difference with regard to the parameters studied but the largest glomeruli were found at juxtamedullary sites It does not appear to matter whether tissue specimens for use in a normal material are removed as small wedges of tissue or as aspiration biopsies Compressed glomeruli near the edge of the specimens should be excluded

Renal biopsies are being used more and more and has increased the need for a *quantitative* evaluation of glomeruli, since it is not uncommon for biopsies to contain alterations which are difficult to evaluate with light microscopy In addition a quantitative method to be used in the study of glomeruli would be helpful when a greater knowledge of the character of changes in glomerular structure and of the progression of such changes is to be gained

The introduction of a *semi quantitative* method has been of great importance in the evaluation of renal biopsies and has lead to a greater precision in the description of lesions and to a greater uniformity in assessment (*Muehrcke et al* 1957 *Pirani et al* 1964 *Pirani* & *Salinas Madrigal* 1968) The method contains however, a subjective element and should be replaced by a truly quantitative method of study Semi quantita

Received 7 vii 71

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tive methods can only be employed when changes are of a certain severity and thus do not reduce the need for a quantitative method for the evaluation of biopsies with minimal changes and the *recognition of* normal glomerular structure

Until *Brun et al* in 1965 published their study of renal biopsies from patients with rheumatoid arthritis, quantitative glomerular studies had included only determination of the number and size of glomeruli In this study, measurement of glomerular area and enumeration of the glomerular nuclei were undertaken for the first time and the previous view that glomerular hypercellularity was present in patients with rheumatoid arthritis could not be supported Furthermore, an irregular distribution of nuclei (localized hypercellularity) was demonstrated in some patients

The introduction of differential counts of glomerular nuclei and determination of mesangial area was of fundamental importance in the quantitation of glomeruli Results of such studies were published in 1968, more or less simultaneously by Kimmelstiel and co workers and by Wehner (*Iidaka et al* 1968, *Wehner* 1968 a, *Wehner* 1968 b) Only a few papers have appeared in which mesangial area determination and/or differential counts have been employed but they have all pre ... ed informations which could not be ... by conventional microscopy (*Fukuhara* 1968, *Kawano et al* 1969, *Wehner et al* 1970 *Wehner* & *Anders* 1970)

A knowledge of the variation of the parameters studied within individual glomeruli between a larger number of glomeruli and between various macro loci in the same kidney is of importance when a quantitative method is used In the following these variations will be referred to as intraglomerular, interglomerular and interfocal variation respectively

MATERIAL AND METHODS

Renal tissue from a 47 year old woman with no history of renal disease who had died of a ruptured intracranial aneurysm was used There were normal urinary volume, normal serum creatinine and no proteinuria Both kidneys were removed immediately after death The right kidney was used as a renal transplant while the left kidney was used in the study reported here The kidney appeared normal both on macro- and microscopic examination except for a 2×2 cm area of recent infarct Tissue from this area was not used Tissue specimens were removed at random either as aspiration biopsies or as small wedges of tissue approximately 1 mm in thickness and then fixed in Carnoy's fluid for 30 hours For supplementary studies, tissue specimens were removed after the kidney had been stored in 4 per cent aqueous formaldehyde for about two months

Paraffin sections were cut with a microtome adjusted to 2 μ and stained with periodic—Schiff—haematoxylin By focusing on the upper and the lower surface the thickness of the sections was determined numerous times and was in almost all cases found to be between 2 and 3 μ This variation in thickness is of no significance for the number of nuclei counted (*Brun et al*) Sections thicker than 4 μ should not be used for differential counts as the position of nuclei in relationship to the capillary basement membranes and the mesangium cannot be accurately determined in such sections

The method is based on camera lucida drawing and planimetry Glomeruli are projected, using a total linear magnification of $\times$ 1350 (oil immersion) unto the table in front of the microscope and the inner limit of Bowman's capsule, the basement membrane of the capillaries and the mesangium are traced out Nuclei of mesangial endothelial and visceral epithelial cells are *noted* and identified on the basis of their position in relationship to the mesangium and the basement membrane of the capillaries (Fig 1) The total area of the glomerulus and the mesangium is determined planimetrically All areas were measured at least twice

RESULTS

Intraglomerular variation In order to eluci ...

whole glomerulus and four glomeruli from

Fig 1 Camera lucida drawing of a normal glomerulus Mesangium shaded mesangial *nuclei* stri ated endothelial nuclei granulated and *epithelial* nuclei black Micrograph of the same glomerulus Periodic—Schiff—haematoxylin



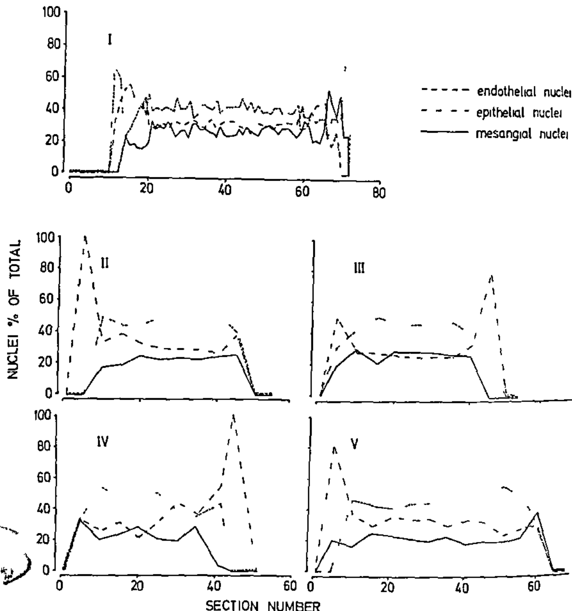
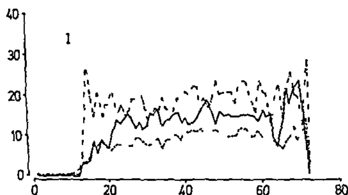


Fig 2 Intraglomerular variation of mesangial endothelial and visceral epithelial nuclei One glomerulus studied in all sections and four glomeruli for each fifth section

the same tissue specimen were studied for each fifth section As seen in Fig 2 a broad central part of the corpuscle reflecting relatively slight variation in the results of differential counts was found in all five glomeruli A similar study was undertaken of the same glomeruli as regards the mesangial area expressed in percentage of the total glomerular area the total number of nuclei per 1 000 μ of total glomerular area and the number of mesangial nuclei per 1 000 μ of mesangial area A graphical illustration of the findings (Fig 3) shows a central part of the corpuscle with relatively little variation as also reflected in the results of the differential counts *In so* far as all the parameters are concerned, *this*

- - - mesangial nuclei per 1000 μ^2 of mesangial area
——— mesangial area as % of total area
-·-·-· total nuclei per 1000 μ^2 of total area



II
III
IV
V
0
10
20
30
40
0
20
40
60
SECTION NUMBER

Fig 3 Intraglomerular variation as regards mesangial area in percentage of total glomerular area, total number of nuclei per 1 000 μ^2 of total glomerular area and mesangial nuclei per 1 000 μ^2 of mesangial area

TABLE 1 *Results of Quanti*

	No glomeruli	Total area μ^2	Mesangial aera μ^2	Mesangial ae % of total ar
I Biopsy	25	11 858	1205	10 2 (1 5)
II Biopsy	15	12 890	1258	9 8 (1 4)
I Wedge	44	12 318	1182	9 6 (1 6)
II Wedge	57	13 772	1333	9 7 (2 0)

Standard deviations are in parentheses

TABLE 2 *Results of Quantitative Anal*

	No glomeruli	Total area μ^2	Mesangial aera μ^2	Mesangial aera % of total area
Subcapsular glomeruli	43	15 230	1397	9 2 (1 4)
Juxtamedullar glomeruli	22	18 583	1663	9 0 (1 8)

Standard deviations are in parentheses

area can be seen in the figures to include between half and almost two thirds of the individual glomeruli

On the basis of the above mentioned s, it seemed reasonable to use serial ions in the further study of interglomeru lar and interfocal variation and to study only such glomeruli as were encountered at least 20 sections from the first or the last section

Interglomerular variation In the study of interglomerular variation, *141* glomeruli from four randomly chosen Carnoy fixed tissue specimens were used Two specimens were taken with the aspiration technique and two were removed as small wedges All well preserved glomeruli were studied when as mentioned above, these were encountered at least 20 sections from the first or the last section which contained portions of a glome rulus Sections including a vascular pole were only used if other sections were not suited for study Mean values and standard deviations for the four tissue specimens are given in Table 1 The results obtained in all 141 glo meruli studied were placed in histograms (Fig 4) All distributions were also tested graphically for normality by means of prob ability paper and a reasonably good approxi mation to a normal distribution was found in all cases

Interfocal variation In order to elucidate interfocal variation the four tissue specimens described above were employed A compar ison of the two biopsies using Student s + test showed no significant differences in percent age of mesangial area differential counts or in number of nuclei per 1 000 μ of total area With regard to number of mesangial nuclei per 1 000 μ^2 of mesangial area on the other hand there was a statistically signifi cant difference between the two tissue biop sies ($p < 0 01$)

A comparison of the two tissue wedges showed no significant differences in percent

...alysis of Four Tissue Specimens

...o. of total nuclei	Mesangial nuclei % of total nuclei	Endothelial nuclei % of total nuclei	Epithelial nuclei % of total nuclei	Total nuclei per 1 000 μ^2 of total area	Mesangial nuclei pr 1000 μ^2 of mesangial area
93	24,9 (3,8)	43 6 (3,6)	31,5 (4,5)	7,9 (1,1)	19,6 (2,5)
100	26,3 (4,3)	42 2 (3,4)	30,7 (4,6)	8,0 (1,1)	21,8 (2,3)
98	25,4 (3 2)	43,6 (4 0)	30,9 (3,5)	7,9 (1,0)	20,8 (2,4)
96	26,7 (3,6)	42,4 (3 2)	30,9 (3,8)	7,1 (1,0)	19,5 (2,2)

Subcapsular and Juxtamedullar Glomeruli

No of total nuclei	Mesangial nuclei % of total nuclei	Endothelial nuclei % of total nuclei	Epithelial nuclei % of total nuclei	Total nuclei per 1 000 μ^2 of total area	Mesangial nuclei pr 1000 μ^2 of mesangial area
91	24 8 (3,4)	43,3 (3,4)	31,7 (3,2)	6,0 (0,7)	16,3 (1,7)
113	24,4 (3 2)	44,5 (3,4)	31,1 (3 8)	6,1 (0,7)	16 8 (2 3)

age of mesangial area and differential counts, but there were significant differences in total number of nuclei per 1 000 μ^2 of total area ($p < 0\ 001$) and number of mesangial nuclei per 1 000 μ^2 of mesangial area ($p < 0\ 01$)

No significant differences in percentage of mesangial area, differential counts and number of mesangial nuclei per 1 000 μ^2 of mesangial area were found by comparison of the two wedges and the two biopsies, but the difference in number of nuclei per 1 000 μ^2 of total area was significant ($p < 0\ 02$)

In order to compare subcapsular and juxtamedullary glomeruli, two tissue specimens were removed parallel with the renal surface corresponding to the same pyramide One was removed from just under the capsule and one from the juxtamedullary area The results are given in Table 2 Any significant differences in the above mentioned parameters were not found With regard to glomerular size, the mean was greatest for the juxtamedullary tissue specimen and the seven largest glomeruli were all found in this specimen Since these tissue specimens were fixed in aqueous formaldehyde the results are not comparable with the Carnoy-fixed specimens

DISCUSSION

The present study of intraglomerular variation reveals that in a series of sections through a glomerulus, a central part is present and found to comprise approximately the middle 50 per cent of the glomerular sections in which variation in the parameters studied is relatively small as compared with the first and last sections in the series of sections Using sections from this central part, histograms of the interglomerular variation show a reasonably good approximation to a normal distribution as regards all statistically treated parameters Concerning differential counts of nuclei, the mean values obtained

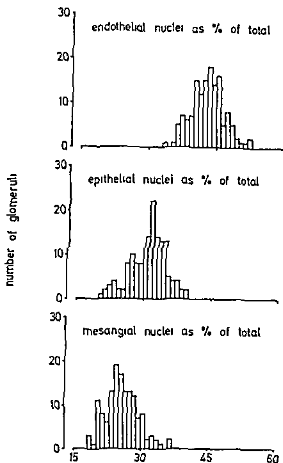
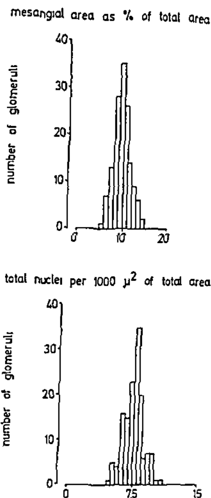


Fig 4 Histograms of 141 glomeruli as regards differential counts of nuclei, mesangial area as percentage of total area, total nuclei per 1 000 μ^2 of total area, and mesangial nuclei per 1 000 μ^2 of mesangial area



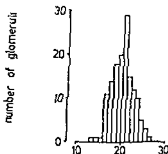


Fig 4 continued

study are very close to those found in the normal material studied by Kimmelstiel and his group who used exactly the same technique (*Iidaka et al Kawano et al*) Differences between the results obtained in the present study and in Wehner's study of a normal material were markedly larger with regard to differential counts This can be explained by the fact that Wehner used all glomerular sections for study regardless of their relationship to the first and last section in the series (*Wehner* 1968 b, *Wehner* & *Anders* 1970)

The fact that a study of interfocal variation revealed significant differences between the two biopsies as regards number of mesangial nuclei per 1 000 μ^2 of mesangial area between the two wedges as regards total number of nuclei per 1 000 μ^2 of total area and number of mesangial nuclei per 1 000 μ^2 of mesangial area and finally between the two biopsies and the two wedges as regards

number of nuclei per 1 000 μ of total area suggests a number of considerations The reason for significant differences with regard to nuclear density could be a) the method, b) a real interfocal variation, c) nonuniform effect of fixation It does not appear likely that differences could be due to the method, since identification of nuclei, even though associated with some uncertainty, must be considered to be just as accurate as dif ferential counts and determination of the me sangium where significant differences were not in evidence. To what degree the ob served significant differences are due to a real interfocal variation or whether the effect of fixation has been slightly non uniform can not be determined The consequence of both situations must be however, that it should be realized before normal tissue for control studies is chosen that it is better to study a few glomeruli in several tissue specimens from the same kidney than many glomeruli from a single specimen If the real variation in results is not registered the posibility exists that false positive results may be achieved in studies of diagnostic renal biopsies

The fact that the largest glomeruli are found at juxtamedullary sites *(Elias & Hen ning* 1967) could lead to the supposition that differences with regard to other parameters could be demonstrated between subcapsular and juxtamedullary glomeruli The absence of significant differences as regards statistical ly treated parameters measured in one tissue specimen with subcapsular and in one with juxtamedullary glomeruli does not of course indicate that such differences cannot be pre sent but the study suggests that such dif ferences are not present systematically

According to a comparison of the results obtained in the Carnoy fixed tissue wedges and in biopsies there is apparently no evi dence suggesting that tissue specimens to be used as control must be obtained by a biopsy technique in order to be compared with biop sies from patients On conventional micro scopy using a high magnification we have occasionally had the impression that certain glomeruli near the edge of the specimen contained a rather large mesangial area On closer examination this appears to be due to a compression of capillary loops Glomeruli of this type should be regarded as damaged and should not be used in quantitative studies, since the mesangial area in these cases will be evaluated as being too large and endothelial nuclei may be mistaken for me sangial nuclei

The present study has demonstrated that a central part in glomeruli is found to com prise approximately 50 per cent of the glome rular area in which there are only slight variations as regards mesangial area in per centage of total area, differential counts total number of nuclei per 1 000 μ of total area and number of mesangial nuclei per 1 000 μ^2 of mesangial area With regard to interglo merular variation, all parameters showed a reasonably good approximation to a normal distribution Interfocally, significant dif ferences in nuclear density can be de monstrated a phenomenon that should be taken into consideration in the planning of a control study A single study suggests that with the exception of the total glomerular area, there are no systematic differences between subcapsular and juxtamedullary glo meruli It does not seem to affect results whether tissue specimens for quantitative studies are removed as wedges of tissue or as aspiration biopsies Compressed glomeruli near the edge of the specimens should be excluded

This study was supported by grant from the *Danish Medical Research Council*

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Acta path microbiol scand Section A 80, 125–133, 1972

QUANTITATIVE STUDIES ON THE DECAY OF LYMPHOID CELLS DURING THE DEVELOPMENT OF CASEIN-INDUCED MURINE AMYLOIDOSIS

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Experiments were performed in order to study the decay of lymphoid cells in various lymphoid organs during the development of casein induced amyloidosis in mice. Furthermore the decay was studied after acceleration of the amyloid formation by nitrogen mustard The decay was calculated on the basis of the nigrosin dye exclusion test on lymphoid single-cell suspensions A significant increase of decay was observed early in the induction phase, in the spleen and in the thymus The decay in the axillary lymph node (regionally to the site of casein injections) and the mesenteric lymph node was unaffected by the treatment During acceleration of the amyloid formation the decay of lymphoid cells was increased in all organs examined The results indicate a relationship between the decay of pyroninophilic cells and the formation of amyloid

The depletion of lymphoid cells is a constant phenomenon in the course of casein induced murine amyloidosis, especially in those lymphoid organs where the amyloid is formed This depletion of lymphoid cells is even more striking when the amyloid formation is accelerated by various lympholytic agents (4, 12 23, 24) Therefore it has been suggested that lymphocyte decay in general is of significant importance to the pathogenesis of amyloid formation (20)

The aim of the present work was to study the rate of cell decay in various lymphoid organs during the development of the casein induced amyloidosis as well as during acceleration of casein induced amyloidosis with nitrogen mustard

The decay of cells was studied on lymphoid single cell suspensions from thymus, spleen, mesenteric lymph node and axillary lymph node, by means of the nigrosin dye exclusion technique (D E T)

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MATERIALS AND METHODS

The animals were randomly selected mice from an inbred colony of the C3H strain Sex distribution was equal, and all mice were 9 weeks old at the day of sacrifice The mice were divided into groups according to treatment (see Table 1)

Experimental Animals

The animals were treated with daily injections of 0 5 ml 5 per cent solution of sodium-caseinate for up to 29 days Within this period the animals

were sacrificed at different time intervals, and lymphoid organs were examined as described below In order to accelerate the casein induced amyloidosis groups of animals pretreated with casein for 17 days received varying doses of nitrogen mustard (Erasol[R]) subcutaneously, and their lymphoid organs were examined as described below

TABLE 1 *Experimental Design*

Number of casein injections	Number of nitrogen mustard injections	Number of mice
0	0	12*
0	1	10§
0	2	5
0	3	5
5	0	13
11	0	10
17	0	8
17	1	10§
17	2	5
17	3	5
23	0	7
29	0	5

* This group—the control group—consisted of animals treated with increasing doses of saline

§ Half the number of animals were injected with a LD_0 and the other half with a LD_{10} The animals treated with repeated doses of nitrogen mustard received a LD_{10} dose per day Animals were killed the day after the last injection

Control Animals

Parallel to the casein injections the control animals received injections of isotonic saline In that of the experiment where nitrogen mustard was plied the control animals likewise were treated isotonic saline prior to nitrogen mustard treatnt

Dye Exclusion Test

All animals were killed by cervical dislocation and the thymus the spleen, the mesenteric lymph node and the axillary lymph node (regionally to the casein injections), were dissected and transferred to chilled Hank s solution Preparation of the individual lymphoid cell suspensions and the nigrosin dye exclusion was performed as described in detail elsewhere (6) This suspension technique ensures that 98 per cent to 100 per cent of the suspended cells are free lymphoid cells with only an insignificant amount of fixed cells such as fixed macrophages and reticulum cells

Histological Preparations

Small parts of the lymphoid organs were fixed in 10 per cent neutral formalin and embedded in paraffin Sections were cut 5 microns thick and stained with haematoxylin eosin methyl green pyronine, alkaline Congo red and the P.A S stain. Amyloid was identified by its morphology and by its birefringence with Congo red under crossed polars

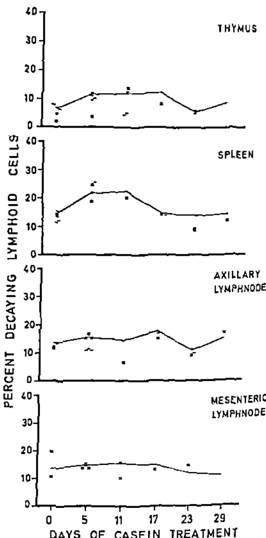
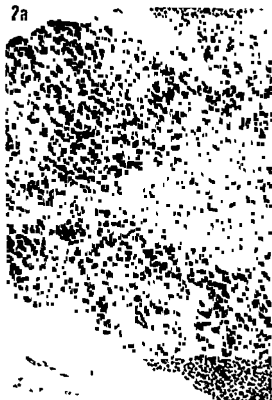


Fig 1 The percentage of decaying cells in single cell suspensions from various lymphoid organs following repeated injections of casein

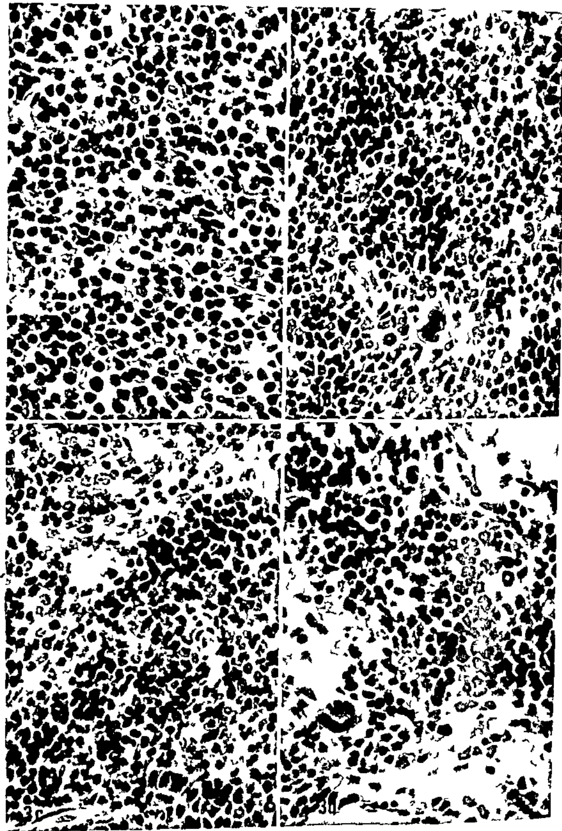
Fig 2 Thymi from animals receiving 0 (a) 5 (b) and 29 (c) injections of casein Note the normal architecture of the organs especially the dense cortex and the sharply marked cortico-medullary border d Pretreatment with 17 injections of casein followed by 3 injections with nitrogen mustard (LD_{10} per injection) Note the complete involution of the thymus with a cortico medullary conversion H & E × 210


2a


2b







RESULTS

Fig 1 shows the decay in single cell suspensions from thymus, spleen, axillary and mesenteric lymph nodes expressed as percentages of non viable cells The thymus and the spleen showed a significant increase in the percentage of decaying cells after 5 days of stimulation with casein (Thymus $p<0.01$, spleen $p<0.01$ rank sum test) The cell decay in the thymus remained increased for up to 17 days while the spleen showed increased values for 11 days only In contrast to this the decay in the axillary and mesenteric lymph nodes remained unaffected during the experimental period

The characteristic histological changes in lymphoid organs from mice receiving casein-injections have been described in detail by *Christensen* (1962) Similar histological changes were observed in the present study and summarized below Throughout the injection period the thymus showed a normal histology *i e* a densely packed cortex and a sharply marked cortico-medullary border Fig 2 shows the thymus from animals having received 0, 5 and 29 injections of casein respectively No histological signs of increased cell proliferation or cell death were evident in this organ Five days of casein treatment induced a marked pyroninophilic perifollicular proliferation in the spleen with a simultaneous depletion of the perifollicularly localized small lymphocytes This pyroninophilia as well as the lymphoid depletion was markedly increased in the group which throughout 11 days received casein injections In the 17 days group the pyroninophilia decreased being replaced by an increasing number of P A S positive macrophage like cells Further injections of casein lead to the formation of perifollicular intercellular deposits of amyloid which on day 29 dominated the histological picture with an almost total depletion of small lymphocytes and pyroninophilic cells Degenerating or pyknotic lymphoid cells were found only occasionally in the red pulp of normal spleens, while the casein treated mice—and especially the groups receiving 5 and 11 injections of casein—showed a high degree of cell pyknoses restricted to the cords of the red pulp (Fig 3) This finding might bear relation to the decaying cells observed in the suspensions of spleen cells at these stages The phenomenon was especially pronounced in the spleen from nitrogen mustard treated mice (see below).

Throughout the period of treatment, the axillary lymph node (regional to the site of casein injections) showed enormous enlargement with marked pyroninophilic proliferation The proliferative changes were seen both in the cortex and in the paracortical areas with a tendency to wipe out the normal structure of the lymph node However, no amyloid deposits were found within these lymph nodes Histologically, the mesenteric lymph node was unaffected by the casein treatment

Fig 4 shows the decay of lymphoid cells in organs from groups of mice pretreated with saline or casein for 17 days At day 18 the animals received either a single high dose of nitrogen mustard (LD_{50} group I) or they received one, two or three injections of nitrogen mustard, respectively (LD_{10} per injection groups II, III and IV) Group I showed the highest degree of cell decay in all organs examined In this group the thymus was found to be the most sensitive organ as judged from both percentage of cell decay and histological changes (Fig 2d), No differences were encountered between the saline and the casein pretreated mice with respect to cytological or histological findings Histologically, all the lymphoid organs showed massive signs of cell destruction with myriads of pyknoses and cell debris No amyloid

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Fig 3 Red pulp of the spleens from animals receiving 0 (a) 5 (b), and 11 (c) injections of casein Figures b and c show a high number of pyknotic lymphoid cells localized to the pulp cords between the venous sinusoids Clusters of extruded erythroblast nuclei are also present d Numerous pyknotic cells in the red pulp of the spleen from a mouse receiving 3 injections of nitrogen mustard (LD_{10} per injection) This animal was pretreated with 17 injections of casein H & E × 525

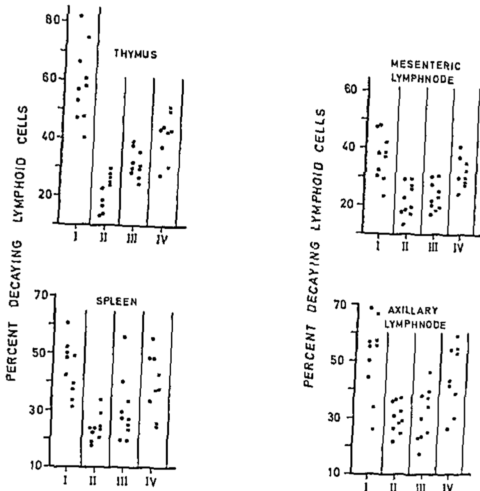


Fig 4 The percentage of decaying cells in single cell suspensions from lymphoid organs following injection of various doses of nitrogen mustard (NM) The animals were pretreated either with 17 injections of casein (□) or 17 injections of saline (●) Group I One injection of NM (LD_{50}) Group II One injection of NM (LD_{10}) Group III Two injections of NM (LD_{10}/injection) Group IV Three injections of NM (LD_{10}/injection)

deposits were found in any of the lymphoid organs of mice in group I

Group II showed only a slight increase in the percentage of decaying cells and no difference between saline and casein pretreated animals was observed The histological picture showed increased numbers of pyknoses in the various lymphoid organs examined No amyloid was observed in this group In groups III and IV increasing numbers of decaying cells were observed in all lymphoid cell suspensions examined Histologically, the spleen showed amyloid deposits in the perifollicular areas with a nearly total depletion of free lymphoid cells in the mice pretreated with casein, while the saline pretreated animals showed lymphoid depletion only Cell fragments and pyknotic cells were dominating features in the lymphoid organs from these two groups As mentioned above the red pulp of the spleen showed numerous nuclear pyknoses especially within the cytoplasm of macrophages localized to the red pulp cords (Fig 3d) No difference between saline and casein pretreated animals was found as far as the percentage of decaying cells was concerned

In all animals receiving nitrogen mustard, the size of the various lymphoid organs was markedly reduced a reduction which ap-

peared to be proportional to the amount of nitrogen mustard injected In group IV the size of all lymphoid organs—both from the saline and casein pretreated animals—were heavily reduced The number of cells in the spleen from a mouse that received 17 injections of casein is about twice the number of cells from a normal spleen Furthermore, the axillary lymph nodes (local to the site of injection) from casein injected mice are enormously enlarged due to proliferation of pyroninophilic cells Thus, the equal percentage of cell decay after treatment of saline injected or casein injected mice with nitrogen mustard, quantitatively represents a higher rate of cell decay in the latter group than in the former one

DISCUSSION

Nigrosin dye exclusion has proved to be a useful tool in the studies of the decay of lymphoid cells in lymphoid organ suspensions and in the blood under a variety of experimental conditions (7, 8) The test evaluates the percentage of decaying cells in single cell suspensions of the lymphoid organ under study Only cells loosely attached to the lymphoreticular mesh of the organ are brought into suspension by the present technique (6) A methodological drawback is however, that the stained (non viable) cell immediately swell thus making further identification of the dead cell impossible The percentages of non viable lymphoid cells in the organ suspension from saline injected (control) animals in the present study correspond closely to the values presented in previous works (6)

Lymphocyte depletion is constantly related to conditions under which amyloid is formed (9 26) the depletion being especially pronounced after acceleration of the amyloid formation with cortisone (23) and nitrogen mustard (24)

The relationship between lymphocyte depletion and amyloid formation has also been suggested in studies of animals developing wasting syndrome either due to a graft versus host reaction (10) or after neonatal thymectomy (11) Furthermore relationship between lymphocyte depletion and the induction times for amyloidosis has been found (9) On the basis of these observations it has been suggested that the decaying of pyroninophilic cells should lead to the formation or release of an inducing substance capable of triggering macrophages into forming the amyloid substance (20, 25)

From cell transfer experiments (19 27) it is known that the time interval between the transfer and the actual formation of amyloid is 4–5 days The increase of the decay of lymphoid cells in the spleen has ceased about 5 days before the spleens enter the amyloid phase This finding therefore supports the hypothesis mentioned above, namely that some kind of an inductor' produced in the pyroninophilic cells is important to the amyloid formation

In the present experiments the significant raise in lymphocyte decay is observed only early in the period of antigen stimulation in the spleen and in the thymus The increased decay in the spleen occuring simultaneous with the increased number of pyroninophilic cells might be explained as an elimination of committed immunocytes by the specific antigen (casein) *Burnet* (1969) suggested this phenomenon to be one of the major events leading to immunological tolerance towards a given antigen Amyloidosis as an expression of tolerance has been proposed by *Cathcart et al* (1970), thus the massive pyroninophilic cell decay in the spleen after 5 and 11 injections of casein might reflect the early steps in the development of a high dose tolerance towards casein In this connection the decay of cells in the thymus 5 11 and 1 days after initiating of casein treatment should be emphasized because of the important role of this organ in the development of tolerance (17 22) An engagement of thymus early in the development of amyloidosis is in line with works on conditions with impaired cellular immunity and amyloidosis (3 21)

The early increase of cell decay in the spleen and in the thymus indicates that the series of cellular events leading to amyloid

formation starts early in the induction phase This is further supported by a recent study (15) showing that spleen cells from donor mice treated for 10 days with casein were nearly incapable of inducing graft-versus host reactions in F_1-hybrids Moreover an early casein induced alteration of spleen cells (pyroninophilic cells) has been found in transfer experiments (14, 16) In these transfer models, cells from mice in the pyroninophilic phase were capable of enhancing amyloid formation in recipients considerably However, such cells were not capable of inducing amyloid formation themselves without further antigen stimulation

The histological changes in the axillary (regional) lymph node were very striking Like others, we found no sign of amyloid formation (5, 9) and the decay of lymphocytes in the stimulated lymph nodes and the more normal lymph nodes (mesenteric) stayed at normal levels throughout the experiments

It is possible that engaged pyroninophilic cells are migrating to the spleen (and the liver) from the locally stimulated lymph nodes Here they might decay, releasing the hypothetical inducing substance as a necessary intermediate step in the formation of amyloid (the "final common pathway" (20) This hypothesis goes well with fact that amyloidosis can be transferred nitrogen mustard treated recipients using dotic spleen cell homogenates, nuclei or even "dead" cells An efferent transport of activated pyroninophilic lymphoid cells from lymph nodes receiving antigenic stimuli to other parts of the lymphoid system has been shown (1 18)

Application of nitrogen mustard gave a significant raise in the lymphoid cell decay in all organs examined, which corresponds to histological examinations made by *Graef et al* (1948) We did not find any difference between the percentage of decay in animals pretreated with casein and the decay in animals pretreated with saline On the other hand, the actual number of decaying cells in the spleen and axillary lymph nodes from mice pretreated with casein and injected with nitrogen mustard is probably twice the number of decaying cells from normal, nitrogen mustard treated mice for reasons pointed out above (p 7). The acceleration takes place after the antigen injections have ceased and lympholytic agents do only cause acceleration if the animals are in the pyroninophilic phase These facts indicate that application of lympholytic agents interfere with the transition between the pyroninophilic and the PAS-positive, amyloid phase

It is striking that the regional lymph nodes failed to develop amyloid during the acceler ation with nitrogen mustard as they contain the postulated necessary elements for amyloid formation *i e* pyroninophilic cells, a heavy decay among these, and macrophages Again it should be stressed that export of lymphoid cells from the lymph nodes might diminish the capability of these organs to form amyloid

During the development of amyloidosis the spleen probably serves as a kind of receptor organ for "circulating amyloid inducing cells" migrating from the regionally stimulated lymph nodes, the primary role of this organ in the pathogenesis of experimental amyloidosis being evident

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EXPERIMENTAL CEREBRAL HEAT LESIONS PRODUCED BY TREPHINE CRANIOTOMY IN RABBITS

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Heat lesions in the rabbit brain were produced by trephine craniotomy with a conventional dental drill The temperature rise was measured at the inner surface of the skull bone at a calculated point over which the burr hole was made A temperature rise of about 10°C was observed to produce an initial and transient injury to the blood brain barrier for about 10 hours, as shown by trypan blue injections Brain edema, apparently produced by an increased vascular permeability, increased in size during the first 48 hours after the craniotomy, as shown microscopically Thereafter the brain edema gradually subsided The heat lesion produced by the trephine burr could be avoided if the area was irrigated with physiological saline at room temperature when the burr hole was made

Brain edema and blood brain barrier (BBB) injuries can be experimentally produced by a variety of means, *e g*, implantations of foreign matter into the brain substance (*Sperl et al* 1957 *Aleu et al* 1964), concussion (*Airo et al* 1952, *Rinder* & *Olsson* 1968), drugs and venoms (*Broman* & *Olsson* 1948, *Steinwall* 1958, *Jeppsson* 1962), hypoxia (*Slobody et al* 1957, *Sato et al* 1969) and cold injuries (*Clasen et al* 1962, *Blinderman* & *Markham* 1965) Heat lesions in the brain have also been observed after ultrasonic radiation (*Barnard et al* 1955, *Åstrom et al* 1961), after placing a heated brass rod on the intact skull bone (*Lee* & *Olszewski* 1959) and after diathermy (*Schmid* 1931)

A common method of performing a craniotomy is through an initially placed burr hole The possible effects of these procedures on the underlying brain tissue, however, have attracted little attention (*Edvinsson et al* 1971) The aim of this study was to investigate in rather more detail the effects of such procedures with regard to the BBB and morphological changes in the adjacent brain

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MATERIAL AND METHODS

Animals – The experiments were performed on 16 adult rabbits of either sex weighing 2 0–3 0 kg

Trephine craniotomy The convexity of the animal s head was shaved and under local anaesthesia (2 per cent lidocain Xylocain Astra Sweden) a 4–5 cm long skin incision was made in the midline over the fronto-parieto occip tal region The exposed area was cleaned from the periosteum over the skull convexity Two or three pairs of burr holes (3 2 mm in diameter) were placed bilaterally to the midline using a dental drill fitted with a special burr (Fig 1) The distance between the centres of the adjacent burr holes was 7–8 mm The burr holes were placed with or without simultaneous irrigation of the adjacent area with physiological saline at room temperature (20°C) The animals were allowed

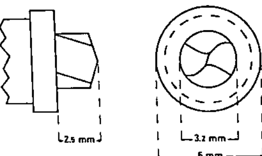


Fig 1 Schematic drawing of the trephine burr used in the experiments The adjustable collar prevents the burr from penetrating through the dura

to survive for periods varying from half an hour to two weeks after the operation

Experimental groups The material was divided into three groups (A, B, and C) with regard to the further operative treatment

Group A The animals were anaesthetised with 6 per cent Nembutal given intravenously (each animal received about 1 8 ml of the solution diluted in physiological saline) within periods varying from half an hour to 14 days after placing the burr holes Trypan blue was given intravenously for 1–3 minutes as a 1 per cent solution in 0 9 per cent NaCl, 15 ml/kg The animals were then sacrificed by intravenous air injections within 10 minutes after the trypan blue injection and the brains were removed inspected, photographed and then fixed in 10 per cent neutral formalin After 3 days the brain was sliced and the slices were photographed After a further 4 days formalin fixation blocks including areas beneath the burr holes were embedded in paraffin and sectioned Every second section (10 μ) was studied by fluorescence microscopy (*Hamberger & Hamberger* 1966) in order to evaluate any damage to the *blood brain barrier* (BBB) The extent of any *brain edema* and other microscopically visible changes were studied by light microscopy after staining the alternate sections in hematoxylin eosin

Group B After the burr holes had been placed the animals were anaesthetised with Nembutal given intravenously at the same time intervals as in group A Under this Nembutal anaesthesia trypan blue was given intravenously as in group A Then a polyethylene catheter was introduced in cranial direction into the right common carotid artery The neck veins were divided and the brain was perfused with physiological saline injected through the polyethylene catheter until the animal died The brain was removed and treated as in group A This rinsing procedure was performed in order to visualize in more detail the extent of the BBB-injuries

Group C Under Nembutal anaesthesia, a mid line incision was made in the skull and the bone was cleaned from periost On the right side near the base of the skull, a temporal burr hole was made and a needle thermocouple (K4 Applicator, Elektrolaboratoriet, København V) was introduced The thermometer was brought into contact with the inside of the frontal or parietal skull bone at a calculated point, over which a burr hole was made The rise in temperature during the burning procedure was recorded on a temperature recording instrument (Type TE3, Elektrolaboratoriet, København V)

RESULTS

A total of 12 burr holes were made in two rabbits (Group C) Eight of these were done during a time course of 5–10 seconds The temperature rise noted was $10 \pm 2°C$ No temperature rise could be observed when the burr holes were made during simultaneous irrigation of the burr hole area

BBB damage – In the Groups A and B, extravasation of trypan blue could be noted promptly after placing the burr hole, but was most evident within the first hour The phenomenon was found to have disappeared in the course of the next 10 hours The circumscribed extravasation of the trypan blue in brain tissue was approximately 2 mm deep and corresponded to the position and size of the burr hole The BBB-damage was of the same magnitude and duration in the animals killed by intravenous air injections



Fig 2 Reactive hyperaemia and brain tissue damage are seen on the brain surface at 6 hours (the most rostral pair of damage areas), 24 hours, and 48 hours after placing the burr holes in the skull bone × 1 5



Fig 3 The microscopic view of the brain edema 24 hours after placing the burr holes × 10

(Group A) and in animals perfused with saline (Group B)

With irrigation of the bone while the burr holes were made, no BBB injury could be detected.

Brain edema – The drilling of the burr hole produced reactive hyperaemia on the brain surface (Fig 2) and a varying degree of [illegible] during an initial increase phase, as [illegible]wn by histological staining in Groups A [illegible] B Within the first 24 hours, the cellular elements, mainly neurons, of the lesion area showed degenerative changes, which made the lesion area easily definable (Fig 3) The nuclei of the neurons showed clumping of chromatin; some astrocytes were enlarged, and their nuclei were pyknotic The blood vessels were markedly dilated and showed a few small perivascular haemorrhages There also occured an inflammatory reaction and after about 2 days necrosis of cortical area was evident

At the end of the increase phase and during the subsequent peak phase, lasting from about 24 to 48 hours the edematous area had reached its maximum size The astrocytes were enlarged and their cytoplasm often showed vacuolizations The adjacent part of the white matter showed separation of the fibres, partly due to vacuoles Haemorrhages were larger during this phase than the bleedings in the increase phase (Fig 3)

During the phase of decreasing edema which lasted from about 48 hours to 14 days, the edematous zone gradually more demarcated and at the end of this period had decreased in size The cell elements, especially those in the gray matter, showed shrinkage Haemorrhages as well as necrotic tissue had been absorbed Only scar tissue was left in the damaged area, producing shrinkage of the brain surface

The time course of the extent of the edema is schematically shown in Fig 4

DISCUSSION

Blood brain barrier – BBB was considered injured when extravasation of trypan blue was noted macroscopically and/or microscopically (*Goldman* 1913 *Becker* & *Quadbeck* 1952) of the cerebral vessels This obser-

freezing of the brain, closely parallels our observations of the amount of microscopically visible edema in the damaged hemisphere (*Clasen et al* 1960)

The BBB injury appeared at the same time as the brain edema This is in agreement with the findings of *Klatzo et al* (1958) that a transient increase in vascular permeability appeared simultaneously with the brain edema However the BBB damage was not demonstrable after 10 hours Thereafter the edematous area continued to increase in size and attained its maximum within a further 15 to 35 hours The edema then slowly began to subside and had disappeared after about 14 days postoperatively leaving only a small scar in the brain surface as described by *Prados et al* (1945)

The absence of any obvious damage to the brain surface when making the burr holes with simultaneous irrigation of the skull bone indicates that the vibrations of the burr itself produced no mechanical trauma to the brain tissue

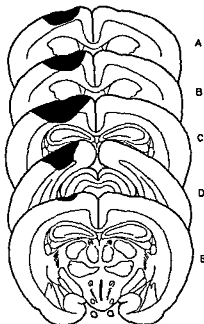


Fig 4 A drawing of the extent of the brain edema at different intervals after placing the burr holes 1/2 hour (A) 6 hours (B) 24 hours (C) 48 hours (D) and 14 days (E) (cf Fig 3)

vation is in accordance with the findings of *Broman* (1949), *Lee & Olszewski* (1959 a and b) concerning the changes in the vascular permeability to trypan blue after air embolism or heat coagulation in rabbits In our experiments there was a peak of BBB damage within 1 hour, indicated by trypan blue extravasation No damage was visible after 10 hours This finding demonstrates that heating produces temporally different effects on BBB than does low temperature where maximum damage as indicated by spread of fluorscein was not observed until 72 hours after focal freezing (*Blinderman & Markham* 1965)

The study of the lesions with trypan blue has revealed a rather striking parallelism to the findings of *Sevitt* (1958) in experimental burns of the skin and of *Clasen et al* (1962 in focal freezing The area stained with Evans Blue was at first localized to the site of the burn or the focal freezing respectively later it extended peripherally This was associated with an accumulation of edematous fluid which for moderate burns and moderate

This work has been supported by a grant (No B71 14X 732 05) from the *Swedish Medical Research Council* and was carried out within a research organization sponsored by the *Swedish Medical Research Council* (projects No B71 14X 712–06A and B71 14X 56–07A)

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Acta path microbiol scand Section A 80, 139–141, 1972

BRIEF REPORTS

SELECTIVE CAPILLARY VASCULARIZATION OF THE NEPHRON IN THE RAT KIDNEY

Poul Faarup, Grete Ryo and Henrik Sælan

The capillary vascularization of the superficial nephron was investigated in rats in which small kidney infarctions—comprising one to several nephrons— were experimentally produced by injection in the renal artery of Sephadex spheres having a diameter ranging from 10 to 100 micron (Fig 1)

A few minutes to several days later, the kidney was vitally stained by the administration of a 5 per cent solution of methylen blue, which was injected in the thoracic aorta A few seconds later, when the total non infarcted tissue, including all the cortical capillaries, was stained, the kidney was rapidly frozen in isopentane at —160° C In the frozen state the infarctions were easily identified macroscopically as small unstained areas in the renal cortex In freeze dried serial sections of the infarctions—in which the sections were orientated parallel to the surface of the kidney—the number of nephrons in the individual infarctions could be counted In these, the glomeruli were surrounded by the occluded proximal tubules Both structures were found to be unstained when the interval between the vital staining and the rapid freezing of the tissue was sufficiently small On the contrary, the glomeruli and the open proximal tubules from the normal kidney tissue, surrounding the infarcted nephrons were deeply stained by the methylen blue (Fig 1) It was further seen that the peritubular capillaries of the infarcted nephron were unstained and dilated clearly different from those of the non infarcted tissue. The lack of stain in the peritubular capillaries of the infarcted nephron thus explains the absent staining of the corresponding tubule A sharp demarcation between the stained and the unstained capillaries was present at the border of the infarction (Fig 2) At the margin of the infarctions the stain could be found to concentrate in the peritubular capillaries (Fig 1), probably due to a decrease in the flow rate in this vascular area Accordingly, when the interval between the in vivo-staining of the tissue and the freezing of the kidney was increased, the peritubular capillaries inside the small infarctions did contain a high concentration of the stain (Fig 3)

The present findings show that the peritubular capillaries belonging to an individual nephron are, to a high degree, restricted to the tubules of this nephron, with no significant overlapping between the vascularization of the different nephrons in *normal* conditions

The results obtained concerning the selectivity of the tubular vascularization in the renal cortex are compatible with the hypothesis of *Steinhausen et al* (1970), concerning the presence of a cortical counter current system between the tubules and the peritubular capillaries Besides, the results fit well with the systematic, correlated orientation of the tubules and the peritubular capillaries found in the renal cortex of the functioning kidney (*Faarup et al* 1971), in which the main axis of the tubules and of the capillaries was orientated radially out to the surface of the kidney in the superficial nephrons

In conclusion, this preliminary investigation has shown that, to a considerable extent *the capillary vascularization of the cortical tubules in the single nephron is functionally restricted to the efferent arteriole from the same nephron*

Received 4 xi 71 from the *Institute for Pathological Anatomy, Bispebjerg Hospital, 2400 Copenhagen*

Supported by grants from *Statens Almindelige Videnskabsfond* "*P A Brandts Legat*" and *Hjerteforeningen*

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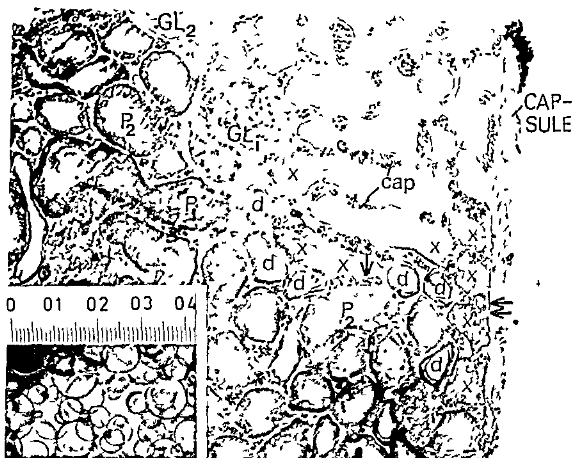


Fig 1 Part of an infarction comprizing two nephrons in which the glomeruli (GL_1 +) as well as the + proximal tubules are unstained The kidney was vitally stained by methylen blue and frozen 3 minutes after the arterial injection of the Sephadex spheres In the infarcted area the corresponding peritubular capillaries are dilated and unstained At the margin of the infarction the loops of the proximal tubule are marked with ↘, and the distal tubules (d) from the adjacent nephrons are placed in the marginal zone Here a proximal tubule (P_2) and some distal tubular loops are partly unstained (single arrow) due to the marginal vascularization from the infarcted nephron Some marginal superficial loops from the proximal tubule in the infarcted nephron have been stained by a few *capsular* capillaries (double arrow)

P_1 first segment and P_2 second segment of the proximal tubule in the normal parenchyme In the first segment the tubular fluid is stained owing to the ultrafiltration of the methylen blue (Freeze dried section × 460)

Inset The size of the Sephadex spheres used are seen from the scale (0 1 100 micron)





loops from the second segment of the proximal tubule in the *figure)* and the corresponding capillaries are deeply stained by methylen blue and distinctly separated *from the* infarcted tissue (Counterstained by osmic acid vapour at the freeze dried state, × 740)

Fig 3 Two nephron infarctions made 3 min before the vital staining by methylen blue which has been maintained for 8 seconds before the freezing of the kidney The infarcted area (in which the occluded proximal tubules are found beneath the dotted line in the figure) is deeply stained as opposed to the unstained glomeruli (GL) The peritubular capillaries are equally coloured In the normal parenchyme, the glomerulus (gl) is stained and in the first segment of the proximal tubule (P_1), the ultrafiltrate is stained as opposed to the second segment (P_2) As in Fig 1 the distal tubules (d) are preferably marginally localized in the nephrons Asc. ascending limb of loop of Henle (Freeze dried section × 460)

Acta path microbiol scand Section A *80*, 142, 1972

TOLUIDINE BLUE EOSIN

A STAIN FOR RAPID DIAGNOSIS

U Henriques

Recently *Desai* (1966) published the results to be obtained when frozen sections were used for diagnostic purposes in cases of breast cancer He used *Terry*'s methylene blue for staining of the sections In this laboratory, his report prompted the adoption of toluidine blue for all frozen sections Soon, however, the need for differentiation was felt Eosin in alcoholic solution was found very satisfactory For rapid diagnosis of cytologic biopsies (fine needle biopsies) with regard to malignancy, as required in peroperative punctures, the toluidine blue eosin gave excellent staining if smears were fixed with Spray fixativ while still wet By now the method is the standard staining method at this hospital, whatever sort of material is sent in for rapid diagnosis

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Received 23 ix 71 from *Institute of Pathology, Central Hospital, Slagelse, Denmark.*

Staining Procedure

1 a smears spray fixativ before drying
1 b biopsies frozen sections
2 toluidine blue 1 per cent, 15 seconds
3 2 3 dips in running water
4 1 per cent eosin Y in 90 per cent alcohol as 1 10, 2 dips
5 dehydration
6 mounting in D P X

Results

Nuclei blue with sharp details, cytoplasm light blue, (or pink in cytology), collagen pink, muscles shades of pink to green, ground substance purple, elastin green, mast cells violet

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Acta path microbiol scand Section A 80, 143–144, 1972

ELECTRON MICROSCOPIC IDENTIFICATION OF ENDOCRINE CELLS IN THE BRONCHIAL EPITHELIUM OF HUMAN FOETUSES

Esther Hage

Cells which are believed to be members of the APUD series of endocrine cells have been demonstrated in the bronchial epithelium of foetal lung (*Hage* 1971). Among the common cytochemical characteristics of APUD-cells were demonstrated: Amine content, amine precursor uptake and reactivity to staining with HCl toluidine blue, Pb H and argyrophilic silver techniques.

The present study is concerned with electron microscopic characteristics of these cells.

Material and method

The material comprised 8 human foetuses, removed by Caesarean section in connection with legal abortion. Crown rump lenght of the examined foetuses were 35 mm, 40 mm, 65 mm, 89 mm, 112 mm, 127 mm, 145 mm and 160 mm. Measurements were made on unfixed foetuses held in a supine position. Fixation: Foetuses were fixed by whole

further by immersion for 3 hours, rinsed in cacodylate buffered sucrose at 4° C (five changes during 1 hour) and stored in buffered sucrose at 4° C for no longer than 7 days. The specimens were postfixed in osmium tetroxid ½ per cent for 2 hours and embedded in epon. The sections were cut on a Reichert OM U2 ultramicrotome. One mμ thick sections were stained with toluidine blue and examined with a light microscope in order to locate areas to be trimmed for thin sections. These sections were stained with Zn uranylacetate 4 per cent and Pb-citrate 0.4 per cent and were examined with a JEM T7 electron microscope.

Received 28.x.71 from *University Institute of Pathology, Odense Hospital, Denmark.*

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Result and comment

In the bronchial epithelium of human foetuses granulated endocrine cells could be observed in the main bronchi as well as in the intrapulmonary bronchi. In the smaller bronchi endocrine cells usually were occuring singly but at divisions of bronchi the cells were often found in groups. The cells (Fig. 1 and 2) were situated basally in the epithelium, all having contact with the basement membrane. The cells were pyramidal or bottle shaped forming a shallow cone which might reach as far as to the lumen of the bronchi. The lateral cell membrane were straight and typical desmosomes were present near the bronchial lumen. Pseudopod like extensions of the basal cytoplasm penetrating deeply into the intracellular space could be observed. The nucleus was generally round and basally located. The cytoplasm was characterized *by the occurrence of numerous secretory* granules uniform in appearance and size. The granules were round, clearly bound by a membrane, and contained a centrally located dense core which was surrounded by a narrow clear space. The granules were generally found in the basal part of the cells. Most cells were poorly granulated but occasionally cells with a great number of secretory granules could be observed. Fixation labile mithochondria, free ribosomes, prominent microtubules and bundles of microfibrils occurred quite regularly. The endoplasmic reticulum was predominantly of the smooth variety, the granular endoplasmic reticulum seemed to be accumulated in flattened elongated sacs frequently found in parallel arrangements. A Golgi complex was seen in a para- and supranuclear site.

The ultrastructure of these cells corresponded well to the ultrastructural characteristics of the APUD series of endocrine cells (*Pearse* 1969).

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Fig 1 Granulated endocrine cell in the bronchial epithelium of human foetus × 26000

Fig 2 Another section of the same cell demonstrating a pseudopod-like extension of the basal cytoplasm × 76000

Acta path microbiol scand Section A 80, 145–149 1972

INTRACRANIAL HIBERNOMA

Report of a Case

P L VAGN HANSEN and O OSGÅRD

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A case is presented in which an intracranial hibernoma behaving completely like a meningeoma as regards symptoms and macroscopical appearance – was first identified during the postoperative histological examination When the tumour was cut through the surface was found to be brownish yellow and numerous tiny pearls of fat swam onto the surface of the liquid in which the tumour was placed Histologically there were no difficulties in the differential diagnosis against meningeoma. This tumour has not earlier been described in relation to the meninges and the central nervous system

The so called hibernoma has been reported in over 70 cases in literature The existence of this peculiar tumour has been known since early in this century and reviews have been published by *Sutherland et al* (1952), *Genesi* (1955) and *Novy & Wilson* (1966) The most extensive review – including the Soviet literature – was published in 1966 by *Apatenko et al* The Hibernoma is believed to arise from brown adipose tissue and relevant information has been published by several authors (4, 7, 9, 10, 14, 16) Of special interest are the histochemical investigations performed by *Fawcett* (6), *Cox* (3), *Wegener* (20) and *Apatenko et al* (1) The aim of this publication is to report a case of a tumour with an unusual localization which has not earlier been reported

Received 3 ix.71

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CASE REPORT

The patient was a 33 year-old woman previously well, and with no family history of epilepsia. Four days before admission she suddenly had several left sided Jacksonian fits At the local neurological department she was found to be drowsy with marked left sided hemiplegia and left sided sensory impairment Eye examination showed incipient papil oedema and a right sided carotid angiography showed a semicircular contrast accumulation in the posterior, parietal region near to the midline and a shift to the left of the anterior cerebral artery and the central vein – indicating a space occupying lesion in the right hemisphere – most likely a parasaggital meningeoma (Fig 1) The patient was transferred to the neurosurgical department G Aarhus Municipal Hospital and operation was performed the next day Opening of the dura disclosed a brownish tumour in the parasaggital region It had a dural insertion very close to the superior saggital sinus – but did not invade it. The tumour had an overall well-defined capsule, and was easy to separate from the surrounding brain tissue It recieved its vascular supply partly from two dural vessels, partly from several larger branches from the middle cerebral artery At removal of the tumour it could not be doubted to be a meningeoma The postoperative period was uneventful The patient reecovered completely and was discharged from the hospital on the 11th postoperative



Fig 1 a and b Right carotid arteriogram showing the space occupying lesion in the posterior parasag gital region with pathological vessels Fig 1a a p projection Fig 1b Lateral projection

day The tumour weighed 56 grams and measured 6,0×6 5×3 cm. Macroscopically it was lobulated – looking very much like a meningeoma – but the cut surface was different, being brownish yellow with small irregular areas of dark brown colour The consistency was firm and elastic When the tumour was cut through, numerous tiny pearls of fat swam onto the surface of the saline, in which was placed The whole specimen was routinely ed in 4 % neutral formalin and representative were brought under the microscope as con ional paraffin sections Already the frozen sec tions had raised some suspicion that the tumour might not be a meningeoma Beside the staining methods routinely used in the neuropathological department for tumours – haematoxylin eosin toluidine blue Weil and Van Gieson – the following special techniques were employed Periodic acid Schiff Scarlach red Sudan black and mucicarmine

Microscopically the tumour was completely dominated by vacuolated cells with distinct cell borders The rounded nuclei of these cells were almost always centrally localized (Fig 2) In the haematoxylineosin stained sections occasional groups of cells with intensely eosinophilic stained cytoplasma were seen These cells contained only scanty amounts of fat or nothing at all In some areas large, rounded cysts were found in which pieces of nuclei and other kinds of cell debris were present – suggesting to the observer that several cells might have fused together as a result of some degenerative process. Most nuclei were completely uniform in size and shape Here and there larger grotesque and hyperchromatic nuclei were lying both single and in small groups (Fig 3), but this was no dominating feature Mitoses were not found and no necroses were seen In some areas beginning proliferation of the smallest vessels might be suspected but as the tumour was very richly vascularized it was difficult to evaluate, whether or not this represented a true proliferation

In the PAS stained sections the vascular network was especially well demonstrated (Fig 4) In the Weil stained sections many of the nuclei were seen to contain 1–4 distinct nucleoli In sections stained with Scharlach red the small vacuoles presented themselves filled with fat which was not stainable

lar penetration

DISCUSSION

Wegener (20) demonstrated in 1951 that brown fat tissue could be found at autopsy in many different places in normal human subjects from 20 to 80 years of age This is consistent with a wealth of information in

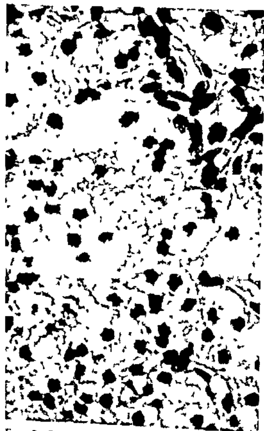


Fig 2 Typical area in the tumour Note the abundance of small vacuoles inside the cells and the centrally localized nuclei (Haematoxylin × 720)

the literature concerning the varying places in which the hibernoma has been found Common locations are between the scapulae, near to the axilla, in the lower postero lateral cervical regions and in the mediastinum Locations in relation to the central nervous system have not earlier been reported *Russel* (15) states that intracranial and intraspinal lipomas are pathological curiosities – nearly all being found in the meninges Lumbosacral localization is relatively frequent in combination with spina bifida In consistence with this, *Lubarsch* (11) writes that lipomas near to the corpus callosum often mean, that this part of the central nervous system is more or less abnormal None of these authors mention the characteristic cells of the hibernoma *Lubarsch* (12) mentions two types of degeneration in meningeomas Fatty degeneration and xanthomatous degeneration In both cases he describes the cells as degenerative in nature with displacement of the nuclei and transitional cell types ranging from normal meningeoma cells to cells heavily loaded with fat and contained in vacuoles, their sizes differing greatly – in contrast to the uniform pic-

[illegible] feature may be augmented in some malignant tumours – e g in some sarcomas Such conditions represent no problems in this case *Solitaire & Krigman* found no hibernoma in a series of congenital tumours (17) Real problems in the differential diagnosis are the liposarcomas, the xanthomas and perhaps the granular myoblastomas Among the liposarc-



Fig 3 Scattered cellular pleomorphism Note the large, grotesque and hyperchromatic nuclei (Haematoxylin × 560

10*



Fig 4 Representative section from tumour Note the rich vascularity and the lobulated appearance of the tissue (*Periodic acid Schiff* × *560*)

-omas, especially the round cell type may semble the hibernomas very much (5, 18) only special fat staining methods are employed these tumours should present no difficulties On the other hand the malignant hibernoma may be impossible to distinguish from a round cell type of the liposarcoma (1) In our case we had no difficulties in the differential diagnosis against the meningeoma which bear only little resemblance to the uniform picture of the hibernoma The scattered cellular pleomorphism and the perhaps doubtful vascular proliferation were the only signs of a possible development into malignancy Some of the cells resembled the 'mulberry cells' described by *Apatenko et al* (1), but this was only seen to a very limited extent We have classified this tumour as benign in type – the relevant literature and the neuropathological findings taken into consideration, but some pathologists may have regarded it as a borderline case to malignancy

CONCLUSION

Whenever a fatty tumour with apparent origin in the meninges is received the neuropathologist must now take the rare hibernoma into consideration as a diagnostic possibility Using special fat staining methods, most differential diagnostic problems will be solved and be of minor importance – except one A malignant hibernoma should always be regarded as a near relative to the round cell liposarcoma

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Acta path microbiol scand Section A 80, 150-158, 1972

PATHOLOGY OF THE HEART FOLLOWING CHRONIC CARDIAC LYMPHATIC OBSTRUCTION

An Experimental Study in Dogs

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The histopathologic effects of impaired cardiac lymph drainage was studied in 22 dogs by excising the cardiac lymph node with its adjoining lymphatics Dye injections and pathologic anatomic examinations were performed from 1 hour up to 9 months after the original procedure Regeneration of major lymph channels was not detected, and only 3 animals showed lymph drainage along alternate pathways Gross distention of subepicardial lymph vessels was present in 5 animals only and was never observed later than after 2 months Hence the observations indirectly suggest the presence of functioning lymphovenous communications The histopathologic changes were confined to the endocardium and atrioventricular valves whereas no abnormalities of the myocardium were detected by light microscopy In the initial stages the subenocardial and valvular lymphatics appeared distended, and from 2 3 weeks onwards a moderate increase in connective tissue was observed in the subendocardial areas Changes were more pronounced in the mitral and tricuspid valves which developed deposits of myxoid substance After 2 months a substantial increase of mesenchymal tissue and fibers was also observed, leading to a marked thickening of the valvular leaflets These changes persisted throughout the observation period and were believed to be of permanent character The observations suggest that impaired cardiac lymph drainage may be of significance to rheumatic heart disease and to myxoid valve degeneration in humans

During the last decade, attention has been focused on the role of impaired lymph circulation in the etiology and pathogenesis of heart disease (7, 8, 11, 14 a o) A variety of ailments may be attributable to lymphatic dysfunction (8), of these, the connection with rheumatic carditis is of particular interest (2, 10) Various surgical procedures also interfere with the lymph circulation of the heart, but the significance for postoperative complications is quite obscure

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Previous studies on lymph circulation and cardiac pathology are few and the results are at considerable variance *Miller and coworkers* (7, 8) reported on hemorrhage and pronounced fibrosis of the endo and myocardium following lymphatic obstruction, whereas *Symbas & al* (13, 14) found very minor changes in these tissues In their experiments the major pathology was confined to the atrioventricular valves

Since the nature, extent and duration of changes after obstruction of the cardiac lymph drainage may have considerable bearing upon heart disease in humans, a

closer investigations seemed warranted In particular, it would appear important to establish whether affection of valves and/or endomyocardial tissue are of permanent character

The present study was designed to investigate changes in cardiac anatomy and histopathology following total obstruction of the cardiac lymph drainage in dogs Investigations were carried out from 1 hour up to 9 months after lymphatic obstruction, in an attempt to evaluate the progress of changes with time, and to decide whether the influence was of transient or of permanent nature

MATERIALS AND METHODS

Young adult mongrel dogs weighing from 18 to 26 kg and fed a standard diet, were selected for the study All animals were anaesthetized with i v sodium pentobarbital (30 mg/kg bw), intubated with an endotracheal tube and ventilated with a piston driven Palmer respirator Cannulas were inserted into the femoral vessels for a slow, constant infusion of Ringer's solution (30 drops/min) and for monitoring of the arterial blood pressure EKG was recorded throughout the experiment. The chest was opened through a left anterolateral thoracotomy in the IVth intercostal space, the pericardium was incised longitudinally anterior to the phrenic nerve and the heart was exposed Multifocal injections of 0 1–0 2 cc of T 1824 dye (Evans blue) were made subepicardially on the anterior and posterior surfaces of all cardiac chambers The areas of injection were screened off with sponges so as to avoid contamination of the parietal pericardium, whose lymph drainage is different from that of the heart proper (5) Immediately after injection a network of subepicardial lymphatics and larger collecting channels was visualized Dissection was then proceeded along the mediastinum exposing the cardiac lymph node between the innominate artery and the superior vena cava. This node was excised together with its afferent vessels from the tracheobronchial node, which is the first recipient of cardiac lymph in dogs (16) A careful search was made for other dye stained lymphatics in the posterior and lower mediastinum including possible tributaries to the thoracic duct Whenever identified such lymphatics were ligated and resected This procedure was performed in a total of 22 animals

Six dogs were subjected to sham-operations, i e injection dissection and identification of lymphatics as described above All lymph vessels and nodes, however were left intact in these animals The edges of the pericardium were then approximated with sutures, the chest was closed in layers with water sealed drainage, and antibiotics (Penicillin G, 4 million units and Streptomycin sulfate, 0 5 g) were administered intramuscularly for the first 4 postoperative days

TABLE 1 *Duration of Lymphatic Obstruction*

Period	Lymphatic obstruction	Sham procedure
1 hour	×	
24 hours	×	×
1 week	××	
2 weeks	×	
3 weeks	××	×
4 weeks	×××	×
6 weeks	×	
2 months	××	×
3 months	××	
5 months	××	×
8 months	××	
9 months	××	×
	21 dogs	6 dogs

Investigations were carried out from 1 hour up to 9 months following interruption of the lymphatics (Table 1) The animals were again anaesthetized and ventilated, and the chest was opened widely through the Vth interspace Pericardial adhesions were partly removed and dye injections were repeated in a similar fashion as originally performed A total mediastinal dissection was then carried out in a search for regenerated lymphatic channels or pathways of aberrant lymph drainage The extent and appearance of the subepicardial network was also noted and photographs taken The animal was then sacrificed, the heart removed while still beating and briefly rinsed in normal saline The chambers were opened and inspected for gross pathologic changes in the walls and valves, whereupon the heart was fixed in buffered formaline (4 per cent formaldehyde in 2 5 M sodium phosphate buffer) Specimens were taken from each atrium, ventricle, the mitral and tricuspid valves and were embedded in paraffin Sections were stained with haematoxylin eosin van Gieson elastin and in some instances with Alcian blue

In 6 of the lymph obstructed animals and in 2 of the control dogs the left heart was opened *in vivo* using inflow and outflow occlusion and injections of small amounts of India ink were made into the free edge of the mitral valve leaflets with the heart still beating When successful, photographs of the mitral lymphatic plexus were taken prior to fixation of the specimen.

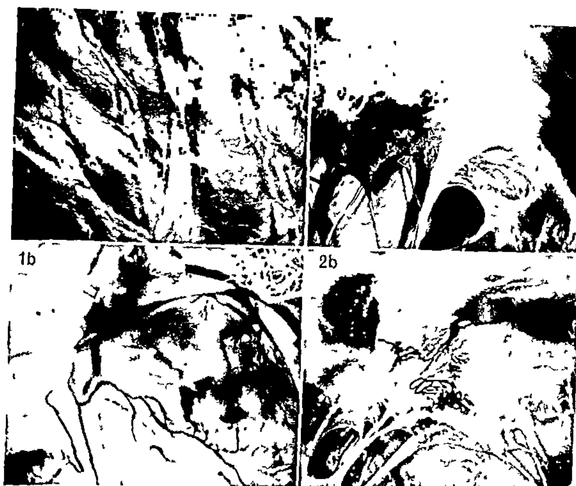


Fig 1 Subepicardial network of lymphatic vessels in a normal animal (a) and 4 weeks after obstruction of the cardiac lymph drainage (b)

Fig 2 Lymphatics in the atrial surface of a mitral valve leaflet in a sham operated animal (a) and 8 weeks after lymphatic obstruction (b)

RESULTS

One of the animals died from an acute pneumothorax on the 3rd postoperative day, leaving 21 dogs in the group with cardiac lymphatic obstruction Two of these animals developed signs of cardiac failure with impaired respiration and peripheral edema The remaining 19 dogs showed no physical signs of impaired cardiac function although their hemodynamic data were indicative of a reduced ventricular performance as compared to the sham operated animals (17)

Macroscopic findings Pericardial adhesions were present to a variable extent in all animals, but there were no signs of infection None of the dogs showed fluid accumulation in the pericardial cavity Dye injections failed to demonstrate lymphatics in the fibrous pericardial adhesions The heart surface appeared normal apart from a moderately thickened epicardium Following dye injections, 5 out of the 21 animals with lymphatic obstruction presented a distended subepicardial lymphatic network (Fig 1) This feature was present mainly in the early periods and never later than 2 months after lymphatic obstruction No such changes were detected in the sham-operated dogs

In all animals, the main lymph channels followed their usual course behind the pulmonary artery to the tracheobronchial lymph node which normally receives the cardiac





Fig 3 Endocardium and myocardium 3 months following lymphatic obstruction The subendocardial layers are moderately thickened (a) whereas the myocardium (M) is of normal appearance Large lymphatic channels (Ly) are present in the subendocardial fibrous tissue (b) van Gieson elastin stain a Left ventricle × 40 b Left atrium × 70

lymph before it enters the cardiac node (16) The tracheobronchial node which had not been excised was heavily dye stained in all animals In the 21 dogs where the cardiac node had been resected no major lymph vessels were seen to drain towards the upper mediastinum In contrast the lymphatic connections in the sham operated group were normal with large lymphatics running from the trachebronchial node to the intact cardiac node whose efferent vessels again drained into the right lymphatic duct

Aberrant lymph drainage was observed in 3 animals subjected to lymphatic obstruction, with large dye stained vessels in the parietal pericardium running cranially to the posterior mediastinum or caudally to the diaphragm Hence the majority of the lymph obstructed dogs showed no aberrant drainage or regeneration of previously resected vessels Lympho-venous communications were strongly suspected but could not be demonstrated with the employed technique (*vide infra*)

There was no evidence of cardiac enlargement or distention of separate chambers No macroscopic changes were detected in the aortic or pulmonary valves in either group of animals The myocardium and endocardium also appeared normal by gross examination The atrioventricular valves, however showed macroscopic pathological changes in all but one animal with lymphatic obstruction of more than 8 weeks duration The valves displayed a yellowish patchy discoloration and



Fig 4 Distended lymph vessels in the annulus of a mitral valve 3 weeks after obstruction of the cardiac lymphatics van Gieson elastin × 70

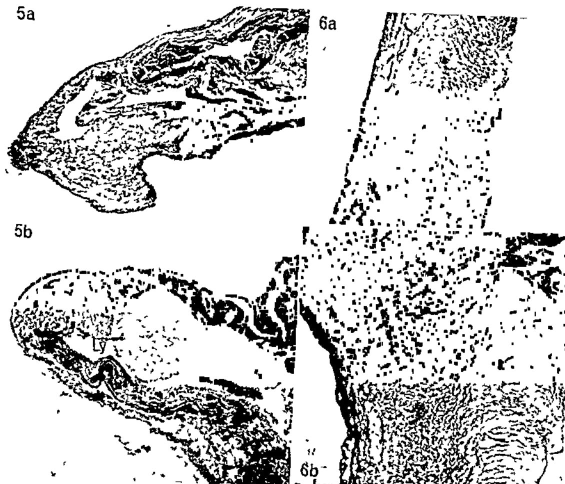


Fig 5 Mitral valve leaflets at 3 weeks showing dilated lymphatic spaces (Ly) Particles of injection India ink are visible within the lumina (b) Hematoxylin eosine × 70

Fig 6 Tricuspid valve leaflets in a sham-operated animal (a) and 5 months after lymphatic obstruction (b) Considerable thickening of the valve is caused by increased connective tissue with deposits of myoxid substance Hematoxylin eosine × 70.

appeared thickened as compared to specimens from the sham-operated dogs These changes as a rule were more prominent in the tricuspid than in the mitral valves Dye injections into the valve leaftlets during cardiac contractions showed an extensive network of distended lymph channels, as compared to the sparse valvular lymphatics in the control dogs (Fig 2).

Microscopic findings

A. *Pericardium* In some animals with lymphatic obstruction the subepicardial lymphatic capillaries appeared more prominent than in the controls The evaluation, however, was unreliable because of the predominant reactive changes with fibrosis and increased vascularity, which were present in both groups of animals and were considered secondary to the operative trauma

B *Myocardium* Very few changes were observed in various sections from the myocardial tissue Occasional small areas of fatty degeneration or fibrosis were present in both groups of animals and a few showed a moderate interstitial edema Hemorrhage, hemosiderin deposits or larger areas of fibrosis were never observed, particularly not in the subendocardial myocardium where such changes were especially sought for The myo-

7



Fig 7 Detail from tricuspid valve leaflet, 8 months after lymphatic obstruction Loose mesenchymal tissue (center) with myoxid (My) interspersed in a fine lacework of fibres Bundles of elastic and colla gen fibres (bottom) towards the ventricular lumen (V) van Gieson elastin × 100

cardial capillaries and larger blood vessels also were of normal appearance as judged from the microscopic observations.

C. *Endocardium* During the first 2 weeks following lymphatic obstruction, the subendocardial lymphatic plexus appeared distended. From 3 weeks onwards, there was also a moderate but definite increase of connective tissue with thickening of the subendocardial area Frequently, larger lacunae of lymphatic channels were present between the connective tissue layers (Fig 3) This pattern was observed in all cardiac chambers throughout the observation period, but was never so marked as to be detected by macroscopic examination. Hemorrhage or pigment deposits were never observed The endocardium of the sham-operated animals was of normal appearance in all instances

D *Atrioventricular valves* These were the sites of the most prominent pathologic changes, which were observed in all the experimental animals and lacking in the controls. From the first hour up to ca 3 weeks after lymphatic obstruction, the predominant finding was a distention of interstitial tissue spaces and lymph capillaries, both in the annulus (Fig 4) and in the valve leaflets (Fig 5) In some of the animals injected with India ink,

TABLE 2 *Sequence of Major Histological Changes following Cardiac Lymph Obstruction*

		1 hour			2 weeks			4 weeks			9 months
Endo card	Distended lymphatics	+	+	+	+	+	+	+	+	+	+
	Subendocardial fibrosis				+	+	+	+	+	+	+
Myo-card	No pathological changes										
M & T valves	Distended lymphatics	+	+	+	+	+	+	+			
	Myxoid deposits				+	+	+	+	+	+	+
	Increased connective tissue							+	+	+	+

dye particles were visible within the lumina (Fig 5b) Over the next 2 months, a considerable thickening of the valve leaflets became apparent (Fig 6) This was due partly to increase of loose connective tissue partly to deposits of amorphous material which stained faintly basophilic with hematoxylin eosine yellow pink with van Gieson and positive with Alcian blue The deposits appeared to be a part of the abundant ground substance of the valve tissue and will in the following be referred to as "myxoid Details from a tricuspid valve with myxoid substance and loose, mesenchymal tissue are given in Fig 7

The above changes were predominant from 3 months onwards and persisted until the end of the observation period of 9 months They were present in both the mitral and tricuspid valves but always more pronounced in the latter Similar changes were not observed in the sham operated animals

A survey of the main histopathological changes and their temporal sequence is given in Table 2

DISCUSSION

Regeneration of lymphatics is reported to take place about 2–3 weeks after ligation (3) but when a length of a lymph vessel is resected the only means of restoring lymph flow is by opening up of collateral communications (1) In the present study only 3 out of animals showed evidence of such collateral or aberrant lymph drainage At the same time the myocardium with its abundant formation of interstitial fluid and production of lymph showed little if any histological evidence of impaired lymph drainage The natural explanation of these seemingly contradictory observations is the presence of lymphovenous communications These are supposed to function under the stress of increased lymph pressure and/or volume (15) The presence and function of lymphovenous anastomoses in the heart has never been investigated and could not be demonstrated with the technique employed in this study A reasonable speculation is that such communications do exist within the myocardium itself e g to the Thebesian veins As opposed to the myocardial lymphatics, the valve lymphatics showed considerable pooling of lymph following the obstruction experiments (Figs 2 and 5), i e in an area of the heart which is devoid of venous capillaries and venules This difference also supports the concept of myocardial lymphovenous communications, and future investigations of this queston certainly seems warranted

Our observations on the endocardium and subendocardial tissue are at considerable variance from those of *Miller & al* (7) who reported on extensive hemorrhages in the subendocardial myocardium during the early stages after cardiac lymphatic obstruction In our experiments no such changes were observed in any of the 12 dogs examined within 6 weeks, whereas a distention of lymphatics and later a moderate fibrosis did occur It is appreciated that pooling of lymph predisposes to laydown of collagen and perhaps of elastic fibers with increase of fibrous tissue components (20 a o) The occurrence of frank hemorrhage on the other hand seems hard to explain on the basis of lymphatic dysfunction alone In the obstruction experiments of *Rusznyak & al* (11) myocardial hemorrhage was observed only when a complete venous obstruction was superimposed by ligating the coronary sinus Our observations up to 9 months after lymphatic obstruction thus fails to support the theory of *Miller & al* (7) that lymphatic dysfunction may be of importance to the development of endocardial fibroelastosis

The main gross and histopathological changes in the present series were confined to the mitral and tricuspid valves Occasional deposits of myxoid substance are known to occur in old animals but never to the extent observed in the present study Moreover the experimental animals were young and in good health and the control animals failed to demonstrate any valve pathology We therefore feel confident that cardiac lymph obstruction indeed does produce abnormalities of the atrioventricular valves and the length of the observation period also indicates that the

changes are of permanent character These observations are in accordance with those of *Symbas & al* (14) in a study extended up to 3 months after lymphatic obstruction, which showed myxoid deposits in the atrioventricu lar valves In addition, our animals also showed an increase of loose mesenchymal tissue, elastic and collagen fibres This was present mainly in the late period following lymphatic obstruction and may represent a second stage in the series of events.

These observations may have a bearing upon cardiac disease in humans and tend to support the proposition (8) that lymphatic dysfunction may be of significance in the pathogenesis of rheumatic heart affections Rheumatic fever is known to affect cardiac lymphatics (2), and there is evidence that the so-called 'Aschoff bodies' are diseased and degenerating lymphatic channels (18) Of related interest is the verification that rheumatic heart affection is associated with de posits of acid mucopolysaccharides in the endocardium, capillary endothelium and ground substance of the myocardial interstitium, particularly around collagen fiber bundles (6) It appears likely that the myxoid substance observed in our and other (13, 14) studies also is composed from mucopolysaccharides similar to those present in rheumatic heart disease

Apart from the few studies on rheumatic carditis (2, 6, 18), investigations on cardiac lymphatic disease in humans are very scarce Recently, however, myxoid degeneration of the mitral valve has been recognized in the floppy valve' syndrome, where the leaflets are abnormally lax and yielding, thus produ cing an isolated, acquired mitral insufficiency (12) Similar changes in dog hearts may produce spontaneous rupture of the chordae with acute mitral regurgitation (9) The descrip tions of the histological changes in such valves appear almost identical with those observed in the present study

There is ample evidence that lymphatic dysfunction does impair myocardial contracti lity in dogs (11, 17) although structural changes have not been detected by light micros copy In humans, lymphatic congestion has been reported to cause myocardial damage with concomitant increase of serum enzymes (4), and cardiac lymphangitis has been associated with conduction defects (10) It has also been suggested that cardiac failure following heart transplantation may be attributable to interference with cardiac lymph drainage (19)

At present it can only be speculated whether impaired lymphatic function is of significance to the arrhythmias and myocardial insufficiency frequently seen after cardiac surgical procedures It does appear, however, that further studies on cardiac lymphatics and lymph drainage are indicated, and that the results may be of significance to clinical medicine

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Acta path microbiol scand Section A 80, 159–168, 1972

AMYLOIDOSIS IN MINK INDUCED BY REPEATED INJECTIONS OF ENDOTOXIN

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Prolonged application of endotoxin derived from *Escherichia coli* led to deposits of amyloid in the spleen and liver in two different genotypes of mink. Simultaneous occurrence of infectious mink plasmacytosis did not seem to influence the response to endotoxin An evident increase in PAS positive reticular cells, situated in close proximity to the amyloid storage, was demonstrated in the liver Such cells were also found in the border zones of amyloid deposits in the spleen, in some cases The results of this study suggest that the amyloid material was produced by "overstimulated" or dysfunctional reticuloendothelial cells

Experimental amyloidosis may be induced in many different ways A common feature in most of the systems used is that the experimental procedure implies a stimulation of the reticuloendothelial system (7) Recently, *Barth et al* (1, 2) demonstrated that amyloidosis could be produced in mice by repeated subcutaneous injections of endotoxin of *Escherichia coli* The amyloid material was found predominantly in the spleen, but in the animals most severely affected, amyloid was also found in the liver and kidneys As amyloidosis is a relatively common condition in mink, the present experiment was performed to elucidate if amyloidosis might be produced in mink *(Mustela vison)* in a similar way A brief discussion of some aspects of amyloidogenesis with relevance to this investigation, is included in the report

MATERIALS AND METHODS

The animals used comprised 20 female mink, 10 of the Sapphire type (Nos 1–10) and 10 of the Standard type (Nos 11–20) The Sapphire mink were obtained from a herd with a high incidence of infectious mink plasmacytosis (IMP), whereas the Standard animals were bred and raised at the Research Station for Fur Bearing Animals, Heggedal, where IMP has been combatted for many years At the beginning of the experiment the animals were approximately 4 months old Pairs of animals were kept together such that one Sapphire and one Standard (Nos. 1 and 11, 2 and 12, and so on), always shared a pen During a period of approximately 2 months all animals received 45 mg *Escherichia coli* 026 B6 endotoxin (Difco Laboratories, Detroit, Mich USA) subcutaneously The endotoxin (lipopolysaccharide) was dissolved in saline at a concentration of 1 mg/ml, frozen at —20° C, and thawed immediately before it was injected The endotoxin was given as a course of 23 injections (three times weekly), the first 5 being 1 mg each, followed by 14 doses of 2 mg, while the last 4 injections each consisted of 3 mg Five additional female Sapphire mink of the same age were given corresponding doses of saline (Nos 21–25) Euthanasia was performed by electric anaesthetization and fracturing the neck 2 days after the last injection As further controls, hepatic and lienal sections from 5 Standard mink of the same age, maintained on the same feed, were studied (Nos 26–30), and, as amyloid deposits in domestic animals show some variability in staining properties, human hepatic amyloid laden tissue was used as control for the staining reactions

Pieces of liver, spleen, adrenals and kidneys were

Received 12 viii.71

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TABLE 1 *Staining Properties of Homogenous Depositions in Liver and Spleen*

Method	Result
H & E	pink
Congo red	pink (birefringence in polarized light)
Methyl violet	red (metachromasia)
PAS	pink to pale violet

fixed in 10 per cent neutral formalin embedded in paraffin, and sectioned at about 5μ The following staining methods were used, either for the demonstration of amyloid or for differentiation from other possible pathological extracellular deposits or normal tissue elements haematoxylin and eosin (H &
acid Sc
toxylin
flavine
of amyloid (14) Hepatic and splenic sections were also stained with methyl green pyronin Unstained

TABLE 2 *Occurrence of Infectious Mink Plasmacytosis and Hepatic and Lienal Deposition of Amyloid*

		Mink No	Lesions indicating infectious plasma cytosis*	Amyloid§ liver	Amyloid§ spleen	Nos of mink with splenic amyloid†
Experimental animals	Sapphire	1	+ + +	—	+ +	7/10
		2	—	—	+ +	
		3	—	+	+ +	
		4	+	—	—	
		5	+ +	—	—	
		6	—	—	+	
		7	—	+ +	+ +	
		8	—	—	+	
		9	—	—	—	
		10	+ + +	+	+	
	Standard	11	—	—	+	6/10
		12	—	+ +	+ +	
		13	—	—	—	
		14	—	+	+ + +	
		15	—	—	+	
		16	—	—	+	
		17	+	—	—	
		18	+ +	+ + +	+	
		19	—	—	—	
		20	—	—	—	
Control animals	Sapphire	21	+ + + +	—	—	0/10
		22	—	—	—	
		23	—		—	
		24	—	—	—	
		25	+ +	—	—	
	Standard	26	—	—	—	
		27	—	—	—	
		28	—	—	—	
		29	—	—	—	
		30	—	—	—	

* According to routine practice in this laboratory the lesions were graded in severity from + to + + + +, based on hepatic and renal changes

§ — is no deposition + + +, + + + are increasing amount of amyloid

† Results are given as positive cases/numbers of mink

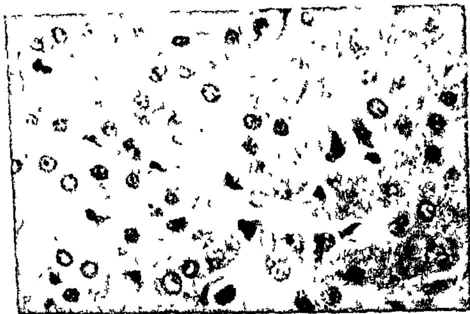


Fig 1 Liver mink 7 Proliferation of PAS positive reticular cells and deposition of amyloid in the spaces of Disse PAS × 400

sections and in some cases unstained frozen sections and frozen sections stained with thioflavine T were examined for birefringence and autofluorescence Pieces of hepatic and splenic tissue from some animals were also preserved for electron microscopy This material was fixed in buffered 2 5 per cent glutaraldehyde postfixed in buffered 1 per cent osmic acid and embedded in Araldite Ultrathin sections from mink 3 and 18 were stained with uranyl acetate and lead citrate

The thioflavine treated sections were examined in a Leitz research microscope ORTHOLUX, with ultra high pressure mercury lamp vertical illuminator (Ploem) excitation filter UV UG 1/2 mm red absorbing filter BG 38/4 mm and barrier filter K 430 Examination for birefringence was performed in a Leitz microscope LABORLUX, supplied with polarizing filters The ultrathin sections were examined in a Siemens Elmiscope 1 A

RESULTS

Growth and Development

None of the animals showed any symptoms after the injections They grew well and developed satisfactorily throughout the experimental period except for mink 21 and 25 which partly lost their appetite and died at the end of the experiment

A EXPERIMENTAL ANIMALS

Autopsy Findings

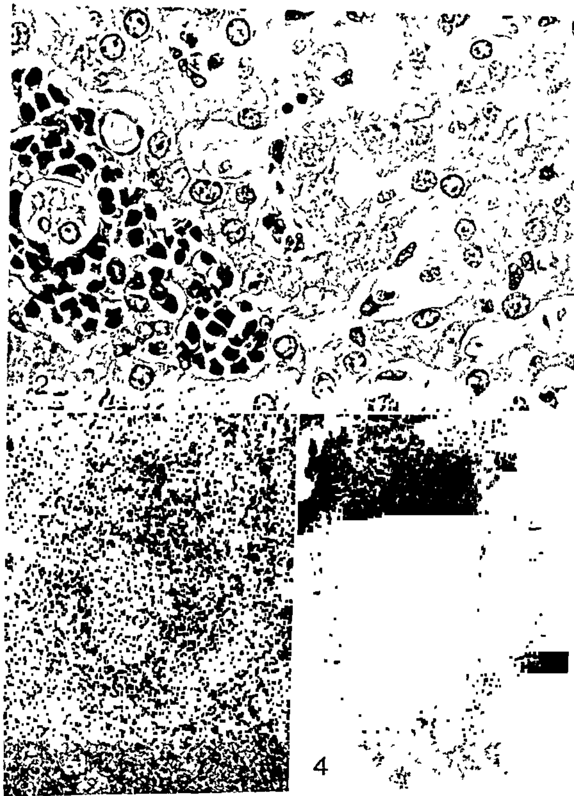
A common description is given for both genotypes as no difference was recognized between the Sapphire and Standard mink

Gross Lesions

The only macroscopic change observed was a variable but usually slight splenic enlargement, and in some animals, a weak yellow brownish discolouration of the liver

Light Microscopic Changes

Liver Homogenous deposits in varying amounts, identified as amyloid by the staining characteristics listed in Table 1, and by examination in ultra violet and polarized light, were found in 6 mink (Table 2) The amyloid deposits were frequently localized in the perilobular areas and were clearly present in the space of Disse Proliferation of reticulo endothelial cells was noted as well, in the cases with amyloid deposits (Fig 1) and in the non amyloidogenic cases The majority of these cells contained PAS positive granules


2
4

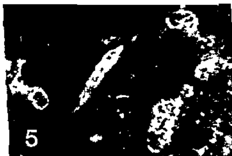




Fig 5 Spleen, mink 7 Fluorescence of the amyloid material in the red pulp, demonstrated in ultra violet light after staining with thioflavine T, × 130

Fig 6 Spleen mink 7 Fluorescence of follicular amyloid demonstrated by the same method as in Fig 5, × 130

Plasma cells, or other pyroninophilic cells, were only sporadically demonstrated in hepatic tissue, except for animals suffering from IMP where circumscribed plasmacytotic accumulations occurred, predominantly in the periportal areas No deposits of amyloid could be recognized in relation to these cells (Fig 2) A usually slight, although somewhat varying infiltration of sudanophilic substance occurred in *the majority of animals*, predominantly in the peripheral lobular areas

Spleen Homogenous deposits with the staining properties of amyloid were found in 13 animals (Table 2) The deposits were present in both the red pulp and in the Malphigian bodies In the former location, the homogenous material accumulated around small arteries, whereas the involvement of the splenic nodules obviously started in the marginal zones of the follicles (Fig 3) or in the juxtafollicular area of the red pulp However, storage was also sporadically seen in the central areas of the follicles, without simultaneous affection of the marginal zones When storage of amyloid was extensive the central follicular area was frequently affected however, usually without involvement of the central artery PAS positive granules were frequently present in reticular cells, and sometimes also in cells morphologically indistinguishable from plasma cells The follicular amyloid deposits were often surrounded by a border of proliferating reticular cells, some of them exhibiting cytoplasmic PAS positive material Degenerating cells and nuclear debris were often observed in the amyloid deposits Considerable numbers of pyroninophilic cells were recognized throughout the red pulp

Fig 2 Liver, mink 18 Proliferation of plasma cells and amyloid storage in the space of Disse Note absence of amyloid within the area infiltrated by plasma cells H & E × 400

Fig 3 Spleen mink 12 Amyloid deposits in the marginal follicular zone Congo red, × 100

Fig 4 Same section as Fig 3 photographed through the polarizing microscope Birefringence of the amyloid material

11



Fig 7 Electron micrograph of amyloid-laden liver, mink 3, a = amyloid fibrils, e = endothelium, p = parenchymal cell, r = reticulin, s = sinusoid Magnification × 30000

In unstained sections, the deposits in the liver and spleen had a rather weak birefringence, in preparations stained with Congo red the pale green anisotropy was markedly increased (Fig 4) Unstained sections examined in ultra-violet light revealed a weak autofluorescence, after staining with thioflavine-T the amyloid showed a very distinct fluorescence (Figs 5–6). A faint fluorescence was also demonstrated in sections stained with Congo red Estimates of the amounts of amyloid in hepatic and lienal tissue are given in Table 2

Adrenal and Kidneys No adrenal or renal changes were detected in the microscopic preparations, except for moderate renal interstitial infiltrations of plasma cells, indicating IMP, in some animals Amyloid deposits did not occur in any animal

Electron Microscopic Changes

Hepatic specimens contained an extracellu-

parenchymal liver cells The fibrils were generally rather haphazardly arranged (Fig 7), the individual fibril being about 100 Å thick A similar fibrillar substance was found in the spleen, sometimes in juxtaposition to reticular cells, but the relation to cellular elements was frequently less obvious than in the liver

B CONTROL ANIMALS

Autopsy Findings

No macroscopic changes occurred in the controls, except for a distinct splenic enlargement in mink *21* and *25* *In these animals*, hepatic and renal lesions indicative of IMP

were present. A variable, and in some cases considerable number of PAS positive reticular cells were observed in the liver. Amyloid deposits were not found in the livers or other organs.

DISCUSSION

The present study demonstrates that prolonged endotoxin challenge in mink leads to storage of amyloid in the spleen and liver whereas none of the control animals developed amyloidosis. Two different genotypes of mink were used in this work because genetic differences were reported to have some effect on murine response to repeated injections of endotoxin (1, 2). Another reason for the use

diak Higashi syndrome (19) which renders the *aa* mink more susceptible than other colour phases to IMP, a slowly progressive disease which is sometimes accompanied by amyloidosis (25, 38).

IMP was first reported by *Hartsough* & *Gorham* (12). As the Aleutian animals are most commonly affected the disorder was originally interpreted as a hereditary disease. It became apparent later that other genotypes could also be affected and it is shown that the disease is transmissible by bacteria free filtrates of infected tissue suspensions (13, 17, 32). Although propagation of the causal agent in cultures of mink tissue has been reported (3) and virus like particles have been demonstrated in infected organs (40) the viral character of the agent does not seem to have been definitely established. Hence the term IMP is preferred in this paper.

Hypergammaglobulinemia and evidence of autoimmune disease have been pointed out by several workers in association with IMP (11, 13, 16, 29, 33). Although bleeding from the mucous membranes is a very common clinical finding severely diseased animals show an enhanced tendency to intravascular coagulation (20, 28) and are highly susceptible to endotoxin and prepared for the generalized Shwartzman reaction (16). The most prominent histological changes are proliferation of plasma cells in various organs, polyarteritis and amyloidosis, as already mentioned, are inconstant findings. Comparisons have been drawn with collagen diseases of man (11) and also with plasma cell neoplasias in other species i. e. multiple myelomas in man and transplanted plasma cell leukemia in mice (9, 10, 25, 29) conditions not infrequently accompanied by amyloidosis. The presence of Bence Jones proteinuria remains inconstant or controversial (29, 39). Plasma cell diseases are very rare in domestic animals other than dogs (26) and mink and amyloidosis in association with plasma cell neoplasias is, as far as the author is aware, only recorded twice in dogs (15, 23) and once in a nutria (18).

Amyloid is classically described as a pale eosinophilic material which may be differentiated from other hyaline substances by certain tinctorial properties. The demonstration of a fibrillar structure of mink amyloid, the widths of the individual fibrils being about 100 Å, is in accordance with the findings in earlier electron microscopic studies in other species (8, 34). An early assumption was that amyloid formation occurred as a reaction between circulating antigen and locally formed antibody, or that the amyloid substance was a precipitate of circulating products of antigen antibody complexes. At present most authors seem to favour the view that amyloid is a product of local cellular activity. Reticuloendothelial cells and plasma cells are among the cell types which have been considered to play a role in the genesis of amyloid. According to *Teilum's* concept (36, 37), the synthesis of amyloid may be explained by "local cellular secretion" that implies initially proliferation of pyroninophilic, reticular cells and plasma cells following protracted stimuli. Secondly these cells are "exhausted", and replaced by a new generation of PAS positive reticuloendothelial cells which ultimately produce amyloid *in situ* (*Teilum's* "two phase cellular theory of local secretion"). However, other authors have found it

more likely that prolonged stimulation of the reticuloendothelial system leads to proliferation both of antibody producing plasma cells, and, simultaneously, proliferation of reticular cells which produce amyloid fibrils, or that amyloid may be the product of plasma cells which are disturbed in their function (4, 7) In the present material, comprising only moderately affected organs, the endotoxin treatment caused proliferation of PAS positive cells in both the liver and spleen As these cells were situated in juxtaposition to the amyloid deposits a relationship with the genesis of amyloid seems likely The occurrence of pyroninophilic cells was not increased in the liver, whilst the splenic red pulp revealed an enhanced number of these cell types It must be remembered, however, that the incidence of IMP was high and that one of the earliest changes in the cellular picture in this disorder is a proliferation of plasma cells in the spleen Accumulation of pyroninophilic cells in the immediate neighbourhood of amyloid storage did not occur in any case, and thus, it must be concluded that the present material was not very suitable for studying a possible relationship between pyroninophilic cells and amyloid deposits, and that such a relationship seems doubtful Furthermore, endotoxin challenge to animals suffering from IMP, did not stimulate plasma cells in the liver to produce amyloid

The manifestation of endotoxin induced amyloidosis may suggest that endotoxin is a factor in the development of amyloidosis secondary to certain chronic infectious diseases A similar mechanism may also be considered in some of the experimental models where living or killed bacteria or bacterial products have been employed Aleutian mink have been shown to be more susceptible to bacterial infections than other colour phases (27), and, in our experience bacterial agents are frequently isolated from the organs of mink succumbing to IMP but as coliform organisms are among the most common bacteria, they are probably often regarded as post mortem invaders Invasion by intestinal bacteria has also been speculated in severe experimental blockade of the reticuloendothelial system (24) Consideration must of course, also be given to the possibility of absorption of endotoxins from autolysing bowel organisms and although considerable discrepancy still exists as to whether or not endotoxins are absorbed from the intestinal tract (5), there is evidence that the stimulus of enteric organisms, or their products, may induce amyloidosis when the immunological apparatus is impaired (22) Delayed hypersensitivity (30, 31) autoimmune mechanisms (35) and immunological tolerance (6) have also been discussed as possible factors in amyloidogenesis In the author s opinion the possibility must be considered, that the occurrence of amyloidosis in connection with IMP may be related to protracted stimulation by low pathogenic bacteria, and mediated by endotoxins or other mechanisms. In any case the reticuloendothelial system is known to play a major role in the defence against endotoxins and invasive agents, and a dysfunctional state must be suspected in animals severely affected by IMP, as their reticuloendothelial system is overloaded or blocked , due to an increased tendency to intravascular coagulation (16) Under such circumstances dysfunctional reticuloendothelial cells may possibly give rise to abnormal products i e amyloid fibrils

In this experiment animals suffering from IMP did not seem to differ from "healthy mink, as far as storage of amyloid in their organs was concerned a finding which possibly supports the hypothesis that factors other than those incorporated in the Aleutian genotypes and/or directly combined with IMP, are involved in the amyloid formation in cases of IMP In this connection endotoxin appears to be one of the more probable factors

In conclusion, it must be pointed out that the amyloid deposits observed in this material were most probably produced by dysfunctional reticuloendothelial cells in liver and spleen, stimulated (or damaged) by endotoxin This stimulation may either be a direct effect of endotoxin on the amyloid elabora

ting cells, possibly mediated by phagocytosis of circulating endotoxin or, as one of the most well known biological properties of endotoxins is to initiate intravascular coagulation, the phenomenon may depend on phagocytosis of fibrin or clotting intermediates A similar mechanism seems possible in amyloidosis associated with IMP, but in this autoimmune disease, excessive phagocytosis of other abnormal plasma components must also be considered On the other hand, the mechanism by which amyloid is formed, may be quite different in amyloidosis associated with IMP and endotoxin induced amyloidosis, as the synthesis of amyloid in animals affected by IMP may be related to a special effect of a subcellular agent on infected cells, as suggested by *Ebbesen* (9) in murine plasma cell leukemia

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The author wishes to thank Dr *A Helgebostad* at the Research Station for Fur Bearing Animals, Heggedal, for providing the necessary facilities for the experimental animals, and Dr *P H J Nafstad* for his help with the electron microscopic work

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Acta path microbiol scand Section A 80, 169-176, 1972

CHROMOSOME ANALYSES OF HUMAN TUMOURS FOLLOWING HETERO-TRANSPLANTATION TO THE MOUSE MUTANT *NUDE*

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Heads H Poulsen and P Christoffersen

The purpose of the present investigation was to study the chromosomal constitution in a number of solid human tumours after transplantation to the mouse mutant *nude*, suffering from thymic aplasia The *nude* mouse was found to have a normal murine karyotype The chromosome analyses were made on tumour tissue, using a direct method without previous cultivation The most important result of the investigation is that only cells with chromosomes of human type were found The majority of the cells analyzed were hyperdiploid, presenting an appreciable variation in the chromosome numbers In some cases it was possible to transplant the tumours in the *nude* mice serially In tumour cells both from early and late passages, only karyotypes which contained nothing but human chromosomes were found Hence no hybridization or total chromosomal species shift was revealed in the heterotransplantation system employed

It has been demonstrated previously that, after inoculation of human tumour tissue on untreated individuals of the mouse mutant *nude*, which suffers from thymic aplasia (10), local tumour growth will occour on the site of inoculation (14) It has been possible to carry out serial transplantation of these tumours Throughout all the passages, the tumours maintain a histological and cytological appearance which is in full accordance with that of the inoculated human tumours (12)

Chromosome analyses were made on a number of the tumours which developed after transplantation to mice The purpose of these analyses was

1 to investigate whether the chromosomal constitution of the tumour cells was of human or murine type,
2 to determine whether the karyotypes presented malignant characteristics,
3 to investigate whether serial transplantation of the tumours studied had given rise to transformation of the chromosomes to murine type

Furthermore, it was of interest *per se* and necessary for carrying out the investigation to determine primarily whether the mouse mutant *nude* had the karyotype which is typical for normal mice

Received 14 xii 71

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MATERIAL AND METHODS

Mice

Mice of the mutant *nude*, five to nine weeks old, bred at the Institute of Pathology, Kommune hospitalet, Copenhagen, (13), were inoculated

Tumour Tissue

The tumour tissue was originally obtained from three patients suffering from adenocarcinoma of the colon and from five patients with metastases from malignant skin melanomas Solid blocks of the human tumour material and tumour tissue from subsequent passages, respectively, were inoculated (see Table 1) The inoculation was performed subcutaneously in the lateral abdominal wall The tumours from the mice were removed for histological study and chromosome analysis, as soon as an intumescence, 8 to 10 mm large (Fig 2), had developed at the site of inoculation, usually after 6 weeks

Chromosome Analysis

The karyotype of the *nude* mouse was determined by chromosome analysis of cells from bone marrow, using a direct method without previous cultiva tion The mouse was given 0 2 ml of an 0 04 % colcemid solution intravenously Four hours later the mouse was sacrified, and bone marrow was aspirated from the thigh and transferred to micro test tubes, containing 2 ml of phosphate buffered (pH 7 0) isotonic sodium chloride solution to which was added 1 microgram/ml of colcemid The suspension was incubated in a water bath at 37 5°C for one hour

Hypotonic treatment, fixation and preparation of slides were carried out applying the methods normally followed in the laboratory (17)

Also the chromosome analyses of tumour tissue from the *nude* mice were carried out by a direct method without previous cultivation The mice were given 0 2 ml of an 0 04 % colcemid solution intravenously four hours before operation A block of tumour was removed surgically, and from this about 30 mg of tumour tissue were used, avoiding necrotic areas as much as possible The tumour tissue was comminuted adding a few drops of a heparinized isotonic sodium chloride solution con taining 1 microgram/ml colcemid

The comminuted tumour tissue was transferred to micro test tubes, containing 2 ml of TCM 199 (Flow) and 0 2 ml of an 0 04 % colcemid solution The suspension was incubated in a water bath at 37 5°C for two hours

Hypotonic treatment, fixation and preparation of slides were carried out applying the methods normally followed in the laboratory (17)

Depending on the quality of the slides, the chromosome analyses were made according to different principles

In cases where the individual chromosomes could be classified definitely, a thorough analysis was made Furthermore, so called screening was performed, i.e., determination of chromosomal type with or without concurrent counting

RESULTS

Chromosome Analyses

Bone Marrow from the Nude Mutant

The slides prepared for chromosome analysis contained rather few cells, and only a limited number of cells were suitable for analysis No numerical or structural chromosome aberrations were revealed All the bone marrow cells analyzed presented normal murine karyotype with 40 telocentric chromosomes (Fig 1)

Transplanted Tumours

The slides prepared for chromosome analysis were normally relatively rich in cells, but in other respects their quality varied greatly Only few cells were found to be suitable for investigation All the mitoses observed in the present material contained chromosomes of human type only The histological characteristics of the tumours and the results of the chromosome analyses are shown in Table 1

It will be seen that complete analysis could be carried out in eight cases in addition to screening in another four cases In only two cases were there no cells of a sufficiently high quality for definite evaluation The distribution of the chromosome numbers found is shown in Table 2

DISCUSSION

Methodology Applied at Chromosome Analysis

The only problem encountered in connection with the technique employed in preparing slides for chromosome analysis from mouse bone marrow is that it can be difficult to aspirate sufficient quantities of bone marrow As mentioned above the analysis of ma

Table 1. Histological Appearance and Chromosome Studies of Heterotransplanted Malignant Human Tumours

o	Patient's record No	Tumour material	Transfer No	Chromosome analysis No	Number of cells analyzed	Chromosome number of cells analyzed	Number of cells screened	Chromosome number of cells screened
1	20246	Well differentiated adenocarcinoma of sigmoid colon Abundant production of mucin	6	450	0	–	4	–
2	–		7	455	0	–	0	–
3	–		8	519	4	51(3), 52(1)	30	32 – 56
4	20956	Moderately differentiated adenocarcinoma of sigmoid colon	1	397	1	48	5	–
5	20961	Poorly differentiated adenocarcinoma of the transverse colon	1	365	0	–	5	–
6	–		2	389	1	45	5	–
7	P2736	Subcutaneous metastasis of left femur from malignant melanoma of left ankle	1	452	1	32	9	34 – 77
8	P2938	Lymph node from right inguen with metastasis from malignant melanoma of right lower leg	1	451	1	44	6	43(1), 45(1), 46(1), 80(1)
9	–		4	529	0	–	5	
10	P2652	Lymph node from left inguen with metastasis from malignant melanoma of left lower leg	3	454	3	59(1), 62(2)	30	33 62
11	–		7	520	1	54	3	49(1), 59(1)
12	P2800	Subcutaneous metastasis from right femur from malignant melanoma of right lower leg	1	542	0	–	2	66
13			3	587	3	62(2), 66(1)	8	43 – 65
14	P3615	Lymph node from right axil with metastasis from malignant melanoma of right infraclavicular region	1	541	0	–	0	–

terial from the *nude* mutant *per se* reveals a karyotype with 40 telocentric chromosomes, which is normal for the mouse

The technique employed in preparing suitable specimens from the transplanted tumours will often be complicated by the fact that it might be difficult to obtain sufficient quantities of vital tumour tissue There are often fairly large necrotic areas in the tumour tissue, and these necroses must be avoided as far as possible when tumour tissue is removed for chromosome analysis Also the appre

Table 2 Chromosome Number of Cells from Heterotransplanted Human Tumours

No	Analysis No	CHROMOSOME NUMBER (32 – 80)
1	450	
2	455	
3	519	
4	397	
5	365	
6	389	
7	452	
8	451	
9	529	
10	454	
11	520	
12	542	
13	587	
14	541	

ciable amounts of colloid material which are found in some of the transplanted colon tumours, may give rise to difficulties, because the tumour tissue contains relatively few cells, and the material tends to get lumpy during the fixation process

In Figs 3–10 histological sections and cells in metaphase from the same tumours are own

As is always the case when solid tumour tissue is to be studied, the analysis takes very long because, generally, only a limited number of cells of a sufficiently high quality are available for analysis

For the purpose of the present investigation it is of the utmost importance to be able to distinguish with certainty between murine and human chromosomes In the literature the murine chromosomes are described as being telocentric or acrocentric (4) We found it justifiable to use the term telocentric because the centromere always seems to be located strictly terminally (Fig 1) A definite distinction between murine chromosomes and human acrocentric chromosomes (groups D and G chromosomes) must be based on the fact that the centromeres of the human acrocentric chromosomes are located subterminally, and that the short arms carry satellites This can be difficult to recognize, and it is a *sine qua non* that only cells of high quality are used The remaining human chromosomes (groups A, B, C, E and F chromosomes) are metacentric and submetacentric and cannot be mistaken for mouse chromosomes However, it should be pointed out that cells from murine tumours

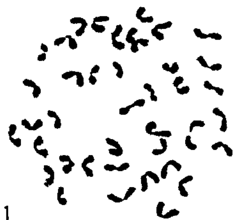
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Fig 1 Karyotype of the *nude* mouse 40 telocentric chromosomes Bone marrow preparation

Fig 2 Tumour bearing *nude* mouse, 4 weeks after inoculation of human malignant melanoma

Figs 3 & 4 Metaphase from and histological picture of a heterotransplanted well differentiated colon adenocarcinoma during the 8th passage Karyotype with 51 human chromosomes (analysis No 519)

Figs 5 & 6 Metaphase from and histological picture of a heterotransplanted malignant melanoma during the 3rd passage Karyotype with 62 chromosomes (analysis No 587)



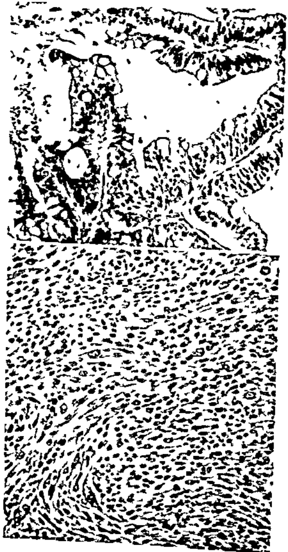
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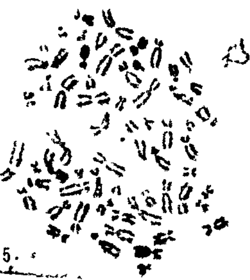
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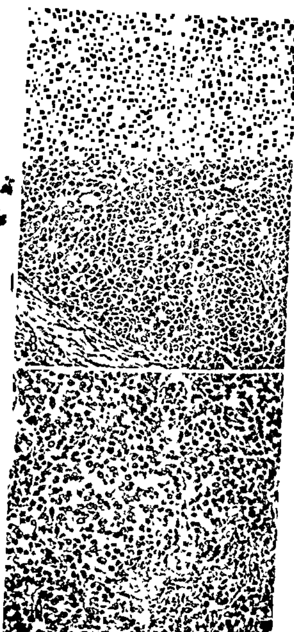
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Figs 7 & 8 Metaphase from and histological picture of a heterotransplanted malignant melanoma during the 3rd passage Karyotype with 59 chromosomes (analysis No 454)

Figs 9 & 10 Metaphase from and histological picture of a heterotransplanted malignant melanoma during the 7th passage (same tumour line as that shown in Figs 7 and 8) Karyotype with 54 chromosomes (analysis No 520)

might contain a *few* metacentric or submetacentric marker chromosomes, which is known *inter alia* from the Ehrlich ascites tumour which is widely used experimentally (*e g. Hasholt et al* (3))

The Chromosome Type of the Tumour Cells

As stated above under Results", only cells with chromosomes of human type were found Chromosomes of murine type were not observed in the analyzed or screened mitoses, nor in the unsuited mitoses observed

The findings prove that the serially transplanted tumours which, histologically and cytologically, are in close accordance with the basic material, are also chromosomally of human type

On the basis of our present knowledge this finding confirms that, in this type of tumour heterotransplantation, it is a question of proliferation of human cells These findings are in accordance with several previous results of studies employing other heterotransplantation systems *(Galton et al* (1), *Haley* & *Strout* (2), *Krishan* & *Raychaudhuri* (6), *Levan et al* (7), *Levin et al* (8), *Miles* (9), *Yohn et al (18))*

Malignant Characteristics

As will be seen in Table 2, the chromosome numbers in the analyzed and screened cells from the tumours vary greatly The majority of the cells are hyperdiploid, only a limited number of cells are hypodiploid The great variation in the chromosome numbers of the cells, and the predominant appearance of cells with chromosome numbers > 46 correspond to the usual findings in non transplanted human malignant tumours There is hardly any better malignancy criterion than such numerical chromosome aberrations The present material is too limited to demonstrate any cloning tendency in the individual tumours (confer analysis No 454, however (Table 2))

The Influence of the Serial Transplantation on the Karyotype

The present material does not allow of any extensive conclusions regarding changes in the karyotype during serial transplantation, as far as numerical changes within the same species are concerned On the other hand, it must be justifiable to state that no hybridization or total chromosomal species shift is encountered

Iversen (5) who carried out serial transplantation of human carcinomas to cortison treated mice found human chromosomal charateristics in the first passage in one case whereas murine chromosomes were found by investigation during subsequent passages The author believed that, during the subsequent passages, the tumour growth might have developed from a carcinogen present in the human transplant Our studies did not reveal anything to support the existence of such a mechanism in our system

Popescu et al (11) transplanted a human melanocarcinoma to mice and rats, using the intra embryonic method By chromosome analyses during the 6th, 7th and 9th passages of the rat tumours developed and of a mouse tumour during the 1st passage, no human metaphases were found among all metaphases analyzed, the constitution of the tumours obtained in rat and mouse presenting the characteristics of the host animal During this investigation, the authors did not make any histological comparison between the basic material and the transplanted tumours

Also these authors mention the possibility of an oncogenetic factor, supposedly of viral origin

Spärck (15) and *Sparck* & *Gross* (16), on the basis of immunological, genetical and histological findings from studies of transplantable mouse tumours, have suggested and found support for the hypothesis that development of tumour growth after transplantation may be the result of activation of primitive mesenchymal host cells which contribute directly to the tumour tissue Our system, in which two different chromosome types are employed, seems well suited to clarify these conditions. The results obtained do not support the existence of such a mechanism under our experimental conditions

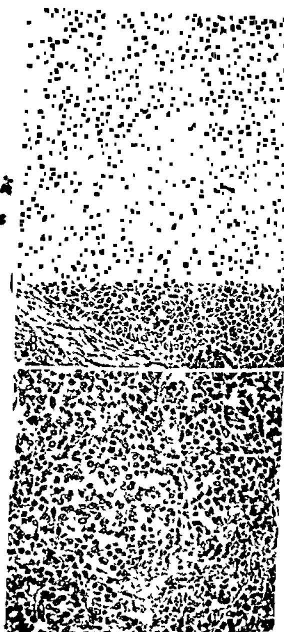
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Figs 7 & 8 Metaphase from and histological picture of a heterotransplanted malignant melanoma during the 3rd passage Karyotype with 59 chromosomes (analysis No 454)

Figs 9 & 10 Metaphase from and histological picture of a heterotransplanted malignant melanoma during the 7th passage (same tumour line as that shown in Figs 7 and 8) Karyotype with 54 chromosomes (analysis No 520)

might contain a *few* metacentric or submetacentric marker chromosomes, which is known *inter alia* from the Ehrlich ascites tumour which is widely used experimentally (*e g Hasholt et al* (3))

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Acta path microbiol scand Section A 80, 177–184, 1972

FREEZE-SUBSTITUTION

A Method Applicable to Routine Surgical Biopsies

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By the method of freeze substitution described, specimens about or less than 2 mm thick and not unduly large in other directions may be processed, in most other methods of freeze substitution smaller pieces of tissue are used Throughout the process the tissue slices are kept in commercial stainless steel tissue baskets Quenching is performed with Freon 22 chilled with liquid nitrogen Freeze substitution for 40 hours at −42 ± 1 C and a subsequent step called final dehydration for 2 hours at about −6 C are performed with ethanol clearing for 90 min with toluene at the latter temperature, all under effective shaking Tissues are embedded in paraffin waxes Different flotation and post fixation methods have been tested The method enables one to use at least some immunofluorescent and some histochemical enzyme methods The morphology of the tissue with a few exceptions is good

Freeze substitution has been surprisingly little used in morphological studies in spite of it offering good preservation of structure (*Simpson* 1941, *Hancox* 1957, *Feder and Sidman* 1958) and that it has been used with good results in immunofluorescent investigations (*Balfour* 1961) and in enzyme histochemistry (*Davis et al* 1959, *Birns and Masek* 1961), it has also given promising results on the electron microscope level (*Bullivant* 1960, *Fernandez Moran* 1960, *Rebhun* 1965) A method applicable to routine pathology should not be unduly expensive nor too laborious, it should be comparatively fast and allow one, with a well preserved morphology, to process tissue pieces large enough to be informative in diagnostic pathology The method presented may fulfill some of these requirements

Received 8 vii 71

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The final method is briefly described in the abstract The different stages were evaluated using a well preserved morphology in H & E sections of rat kidneys and livers as a reference A few general problems, perhaps not hitherto fully recognized, emerged during the work, solutions of these and of the practical problems are discussed in the chronological order of the method The method will probably undergo modifications in the future, at the end of each section there is a detailed description of the way in which the stage at present is carried out in our laboratory Some experiences with different staining methods on human freeze substituted routine biopsies are described under the heading "staining results'

QUENCHING

Very rapid freezing rates are usually regarded as axiomatic in low temperature histologic techniques *Simpson's* (1941) opinions in part constitute an exception, the relative size of his innermost, comparatively well preserved zone enlarged when he

used rather high quenching temperatures Because of this the following methods were tested Acetone/dry ice, isopentane chilled with liquid nitrogen, plain liquid nitrogen, talcum powdered tissue quenched in plain liquid nitrogen or in isopentane chilled with liquid nitrogen (*Molne and Glenner 1964*) and Freon 22 (difluoro-monochloromethane) chilled with liquid nitrogen (*Rebhun* 1965) The results were judged by visual estimation of the freezing times from the cut surfaces of pairs of specimens and by the morphology in H & E sections The fastest freezing rates, apparently also the shortest substitution times and the best morphological results were obtained with Freon 22 *Rebhun* (1965), in his electron microscope studies, seems to have been the first to use Freon 22 for quenching *He regarded it as superior to Freon 12, 13, 14 and even to liquid helium* (*Fernandez-Moran* 1960) despite it solidifying by —160 C Freon 22 boils at —40 C

Technique at present The fresh tissue is put in commercial stainless steel tissue baskets, quenched with Freon 22 chilled by liquid nitrogen (Fig 1), and the baskets are stored in liquid nitrogen until substitution is started The Freon is kept in a metal vessel fitted with a screw cap tightened by a perbuna 0-packing which enables one to re-use the same lot of Freon many times The vessel is guaranteed to withstand the pressure of Freon 22 at temperatures considerably higher than room temperature

FREEZE-SUBSTITUTION

Substitution Medium and Fixation

Absolute ethanol or acetone have been used by most investigators N butanol (*Hancox* 1957) was found very slow, even at —20 C small cubes of ice in n butanol did not decrease much in size in 13 days whereas they melted in 4–6 h in ethanol and acetone Ethanol and acetone seem to extract different substances at different rates (*Ostrowski et al* 1962 *Patten and Brown* 1958) With regard to substitution times and morphology no great differences were found between these two Most investigators have added fixing agents to their substitution media The rate of insolubilization of protein caused by fixatives at low temperatures is said to exceed the rate of inactivation of enzymes and antigens (*Davis et al* 1959) on the other hand osmic acid seems to eliminate the antigenic properties of immunoglobulins and even short post fixation with acetone seems to abolish almost all staining of antibodies fixed in $HgCl_2$ (*Balfour* 1961) It was found *that* satisfactory results may be obtained without *fixation*

Technique at present Substitution is carried out with absolute ethanol without addition of fixatives Sections are post fixed if required

Substitution Temperature

In both freeze substitution and freeze drying, the final results largely depend on the prevention of ice *crystal growth*, both when quenching and during substitution or drying (*Bell* 1952, *Neumann* 1958, *Pearse* 1968) *During the latter*, the temperature has to be kept low enough to prevent ice crystal growth Substitution temperatures below —55 C the eutectic point of $CaCl_2$ (the tissue salt with the lowest eutectic point), might be least damaging but for practical purpose, both *Neumann* (1958) and Pearse (1968), however, seem to regard —40 C as permissible Good morphological results have been obtained at the latter temperature (*Baud* 1952 *Hancox* 1957, *Lison* 1949, *Simpson* 1941) whereas they are considerably poorer after substitution at —30 C (*Simpson* 1941) No light microscopic studies seem to have shown the results to be poorer at —40 C than at —70 C, the latter requires long substitution times and very small pieces of tissue (*Balfour* 1961, *Feder and Sidman* 1958)

Technique at present An apparatus kept at —42 ± 1 C is used Concerning the temperatures during the last stage of substitution, see below under the headings substitution time" and "final dehydration'

Substitution Apparatus

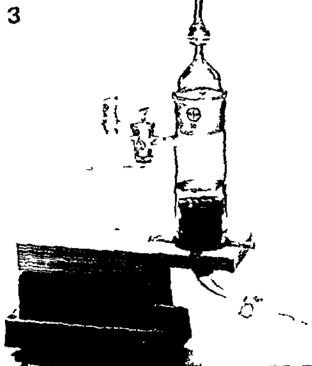
which holds about 3 litres of the substitution medium absolute ethanol The cylinder has a collar

in the lower parts of the cylinder The tray is welded to a copper bar passing through its centre, at the lower end of the bar there is a spring The upper end of the bar is made of insulating acryl plastic and projects above the two-part lid of the substitution cylinder An electro-magnetic device (an air pump from a broken-down *incubator* for premature babies similar the one in Fig 3) vibrating at the 50 cycles sec of our alternating current is put on top of the bar and thus the tissue baskets vibrate at the same speed

1



3



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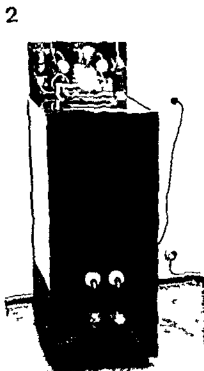


Fig. 1. The tissue in the basket is dropped into Freon 22 chilled by liquid nitrogen. Next the tissue basket is transferred to the liquid nitrogen in the flask.

Fig. 2. The freeze-substitution apparatus with the refrigerator unit in its bottom part. The electromagnetic device on top of the apparatus vibrates the tissue baskets.

Fig. 3. The vibrating apparatus used for final dehydration and clearing at about —6 C.

Substitution Time

When pieces of tissue are removed from the substitution medium at —42 C, it seems very easy to judge from the cut tissue surface how far substitution has advanced, the innermost, unsubstituted area looking like fresh tissue inside the apparently substituted outer zone Substitution of 3 mm thick slices of rat liver seems completed in 24 h. If this really would represent complete substitution it should have been possible to clear such tissues directly with clearing agents which remain fluid at low temperatures (*e g* toluene) It was, however, impossible to obtain easily cut blocks or adequate morphology when such apparently substituted tissue was transferred to toluene at —22 C, and the results did not improve even if substitution in ethanol was prolonged up to 8 days at —42 C, nor by subsequent substitution up to 18 h at about —22 C *Pearse* (1968), in spite of his earlier (1960) doubts, seems to believe in complete substitution at substitution temperatures The results, however, improved when tissues were brought to room temperature in the substitution medium before clearing as done by most investigators (*Simpson* 1941, *Hancox* 1957, *Nilsson and Wirsen* 1960, *Balfour* 1961 *Rebhun* 1965) No great attention has earlier been paid to this last stage of substitution, it seems that it should be separated from substitution proper and it is suggested that it should be called final dehydration

Technique at present Substitution proper is carried out for 40 h at —42 ±1 C

Final Dehydration

In freeze drying according to *Neumann* (1958) 2–4 per cent of the water (Restfeuchtigkeit) is more tightly bound, 'adsorbed to the tissues than the crystalline ice which sublimates easily The tightness of this adsorption is greatly influenced by temperature This may explain the difficulty to obtain easily cut blocks or adequate morphology after substitution and subsequent clearing below —22 C in spite of complete macroscopic substitution The protein denaturing effect of ethanol should be neglible below —5 C (*Edsall* 1947) Consequently after macroscopically adequate substitution at —42 C for 40 h, final dehydration times from 1 to 24 h about —6 C were tested in the vibrating cylinder shown in fig 3 The blocks were easy to cut and they had well preserved morphology even after 1 h of final dehydration It is suggested that substitution proper at —42 C removes crystalline water but not the absorbed water which requires a higher temperature for a short period of time, *i e*, final dehydration

Technique at present After substitution proper the tissue baskets are transferred in about *—20* C ethanol to the dehydratio[n] cylinder (*Fig 3*) containing ethanol chille[d] *to below —6 C* The dehydration cylinder [is] kept in the refrigerator at about —6 C Th[e] final dehydration time is 2 h with one chang[e] of chilled ethanol The temperature neve[r] rises above —6 C The tissue is cleared i[n] the same cylinder at the same temperature

CLEARING AND EMBEDDING

Toluene has a MP of —96 C and clears well at —[…] C Xylene did not clear as well as toluene at thi[s] temperature, perhaps because of the paraxylen[e] (MP 13 2 C) in the mixture Chloroform (*Fede[r] and Sidman* 1958) and cumene (*Balfour* 1961) might be better than toluene, they have not bee[n] tried

Different paraffins (MP 39 56 C) and also ou[r] routine paraffin mixture have been tested with regard both to the morphology and to the demonstration of human immunoglobulins with immunofluorescence, no considerable differences were found Ester wax and 400 polyester wax (*Hancox* 1957, *Balfour* 1961 *Sidman et al* 1961) were not tested *Balfour* (1961) has paid attention to storage problems

Technique at present Tissues are cleared for 90 min with two changes of toluene (about —6 C) in the apparatus used for final dehydration Next the baskets are brought to room temperature in the toluene and embedded in our routine wax (¼ *of* MP 45 C and ¾ of MP 56 C paraffin with $^1/_{15}$ of beeswax added) in a paraffin oven provided with a water suction pump The embedding time is *1* h with *3 changes of* paraffin Before embedding the paraffin is degassed in the oven for 30 min *Valuable* blocks are stored in a refrigerator, others in *cold, dark room*

SECTIONING AND FLOTATION

The sectioning of paraffin blocks with low melting points requires well cooled knives and blocks, our routine paraffin offers fewer problems, it ribbons quite well

Plain tap water as a flotation medium *destroys* the tissue For both routine and other methods 80 per cent ethanol was used earlier, the sections had a well preserved morphology In sections stained for immunoglobulins with FITC coupled *sera or*

in sections stained for nonspecific esterases, however, there were large completely negative areas which did not have the same localization in adjacent sections. They seemed to correspond to areas which had become wetted during flotation. They might have been due to protein denaturation by the 80 per cent ethanol.

For intracellular immunoglobulins 18 per cent Na_2SO_4 (*Balfour* 1961) has been used with good results, the general morphology seems adequate but at present absolute ethanol is used for most purposes. The sections unfortunately tend to submerge in this medium and it is difficult to flatten them. Very small pieces tend to detach from the slides during subsequent deparaffinization and treatment with ethanols. Freshly albuminized slides offer some help. The 18 per cent Na_2SO_4 offers fewer problems in this respect.

Main obstacle at present. A flotation medium suitable for all staining methods and allowing one to flatten sections without fixation of the tissue has not been found.

STAINING RESULTS

Routine and Some Histochemical Methods

Specimen size influences the morphology. In large pieces of tissue with a maximum thickness of 2 mm, *e. g.* across whole large human tonsils, there are sometimes cracks and areas with noticeable ice crystal artefacts but so far it always has been possible to give a usual morphological description of the tissue. Most, but not all, tissues studied in pathological laboratories have a well preserved morphology. Brain tissue always and pieces of skeletal muscle larger than 2 mm in any direction consistently give poor results. It is difficult to cut strongly hyalinized tissues.

For routine staining methods sections are deparaffinized in xylene for 5 min, washed briefly in absolute ethanol and left in another change of absolute ethanol for 20 min and for the same period of time in 96 [illegible]

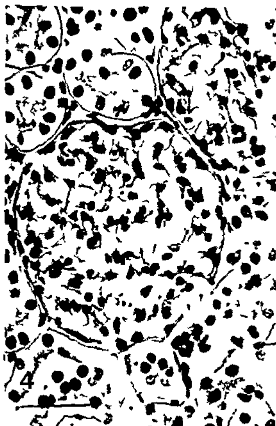
It pays to shorten staining times with haematoxylins. At least the following methods have been used on human tissues with expected results: H & E, Weigert's haematoxylin van Gieson, periodic acid Schiff with and without digestion with saliva (Figs. 4 and 5), toluidine blue, Weigert's resorcin fuchsin, Wilder's reticulin stain, Congo Red for amyloid, a methyl green pyronin stain (Fig. 6) (*Ahlqvist* and *Andersson*, in press) and Sudan Black B.

Immunofluorescent Methods

For immunofluorescence sections are floated on absolute ethanol, deparaffinized with xylene, washed briefly in ethanol, 'fixed' in another change of ethanol for 10 minutes and then brought directly to phosphate buffered (pH 7.2) saline (PBS) with vigorous shaking to avoid the denaturing effect of dilute ethanols. Sera are applied after 2 further washing in PBS. Mostly, FITC coupled antisera (*Wager et al.* 1971) against human immunoglobulin and its subclasses IgM, IgG and IgA in human kidneys, lymphoid organs (Fig. 6) and in various pathological tissues have been tested. Results of such studies will be published later. In some cases sections from freeze substituted tissues have been compared to frozen sections of the same tissue, in the frozen sections it is difficult to obtain a positive reaction for intracellular immunoglobulins of the same magnitude as in freeze substituted tissue. In 14 h a large part of human immunoglobulin is extracted by PBS and even more by $^1/_{15}$ M KH_2PO_4 from sections of freeze substituted rat kidneys, the blood vessels of which are filled with human serum.

Enzyme Histochemistry

Alkaline phosphatases, acid phosphatases and non specific esterases (*Pearse* 1960) may be demonstrated in blocks of freeze substituted rat tissues. As expected (*Ahlqvist and Saikkonen* 1964), non specific esterases were negative in rat ear sebaceous glands in contrast to organs containing less easily extractable lipids. Bile canalicular naphthylamidases which stain intensely in fine needle aspirates (*Wasastjerna* 1969) do not stain as strongly in freeze substituted liver.


4


5


6


7

Immunological studies on tissue sections usually are performed on fresh frozen sections, but in addition permanent blocks of freeze dried and freeze substituted tissues and of tissues prepared according to the method of *Sainte Marie* (1962) have been used All of these four methods have certain draw backs Cryostat temperatures favour the formation of ice crystal artefacts quite independently of quenching rates (*Neumann* 1958) Substance shifts and probably also membrane damage are always present at cryostat temperatures although most employers of the cryostat seem unaware of this, since the artefacts disappear at thawing (*Stowell et al* 1965) Ice crystal artefacts cannot arise in tissues prepared according to *Sainte Marie* (1962) and they are probably of a much smaller magnitude in adequately quenched freeze dried or freeze substituted tissues than in the cryostat Protein denaturation probably increases in the following order Cryostat sections, freeze dried tissues, freeze substituted tissue and tissue prepared according to *Sainte Marie* (1962) The ease with which proteins may be extracted probably decreases in the same order, in the study of immune disorders it is of some importance to be able to extract immunoglobulin which is not bound to its antigen Lipids are preserved in fresh frozen sections, extraction occurs with all the other methods, probably increasing in the same order as protein denaturation In studies on Ig containing cells fresh frozen sections should not *a priori* be regarded as superior to sections prepared by any of the other methods (*Balfour* 1961, *Sainte Marie* 1962, own observations)

The rapid quenching obtained with Freon 22 and liquid nitrogen, the high but probably permissible substitution temperatures and the vigorous shaking used throughout this method of freeze substitution allows one to process pieces of tissue larger than those recommended by most earlier employers of freeze substitution, the time does not very much exceed those in fixation paraffin embedding sequences The tissue need not be brought to room temperature in the substitution medium and this phase, here called final dehydration, may be performed at a temperature (—6 C) at which protein denaturation should be less extensive than with earlier methods of freeze substitution

Today there is a need for methods permitting one to perform some immunological and histochemical enzyme studies, respectively, after a routine histological study has been made Cryostat blocks are neither permanent nor easily handled Of the other methods mentioned, freeze drying seems rather expensive and complicated In these respects freeze substitution probably constitutes as intermediate between freeze-drying and the ingeniously simple method of *Sainte Marie* (1962) Freeze substitution may offer some further advantages especially in regard to protein denaturation and perhaps even in regard to well preserved morphology

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Fig 4 Rat kidney glomeruli and tubuli stained with periodic acid-Schiff and hematoxylin (scale = 50 μm)

Fig 5 Human viral hepatitis The glycogen shows no polarization artefacts in the section stained with periodic acid Schiff and hematoxylin. In freeze substituted biopsies bile seems better preserved than in formalin fixed tissue and the canalicular bile thrombus in the lower left corner is prominent (scale = 25 μm)

Fig 6 Human spleen from a case of Coombs negative haemolytic anaemia stained with methyl green pyronin Most of the pyroninophilic cells close to a trabecula and around small vessels in the red pulp are mature plasma cells (scale = 100 μm)

Fig 7 Section adjacent to that in fig 6, stained for IgM and microphotographed at the same magnification. Many of the cells seem to contain immunoglobulin of this class and to correspond to the pyroninophilic cells in fig 6 (scale = 100 μm)

This study was supported by a grant from the *Sigrid Jusélius Foundation* The fluorescent antibodies were provided by the Municipal Bacteriological Laboratory of Helsinki, headed by *Odd Wager*, M D The technical skill of Mrs *Aino-*

Marja Reims has been of great help during the work

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AMYLOID-LIKE SUBSTANCE SURROUNDING MAMMARY CANCER AND BASAL CELL CARCINOMA

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A particulary substance by the authors termed "elastic amyloid" occurs in cases of mammary cancer and cutaneous basal cell carcinomas The substance displays some properties similar to those of classic amyloid such as staining with Congo-red, responsiveness to methyl violet, iodine green and Lugol's solution It is however not birefringent after staining with Congo-red and is stained by elastica stains A maturation process of the material seems to develop which suggests similarities to changes observed in experimental amyloidosis The relation of the "elastic amyloid" to immunological processes around nests of mammary cancer and basal cell carcinoma is discussed

Recent clinical, patho-anatomical, histochemical and electron microscopical studies have revealed some new types of disorders due to amyloidosis such as familial amyloidotic polyneuropathy (1), amyloidosis in rheumat arthritis, familial mediterranean fever (8, 13) amyloidosis in mongoloids (35 11) as well as in patients suffering from urticaria and deafness (11) Amyloid was also found to be associated with medullary cancer of the thyroid gland (2 39) solid cancer of the pancreas (7) and to occur in organs of ageing people (32 33, 34 35) The results of these studies were discussed at a Congress held in *Groningen* The Netherlands 1967 (22) It was agreed that the deposits observed in all these conditions were formed of the material, which for more than hundred years ago has been known "amyloid"

In previous investigations we described a fibrillar, or finely granular, often amorphous material which surrounded nests of some malignant tumours (30, 31) This substance was stained intensely red in haematoxylin eosin, yellow in Weigert van Gieson preparations Formerly it was termed as "fibrinoid" We demonstrated this substance within and in the vicinity of carcinomas of the rectum, parotid gland, urinary bladder and in mammary cancers, especially in cases of undifferentiated scirrhous tumours, as well as in basocellular carcinomas of the skin

Reuterwall (26) found in spontaneously healing parts of mammary carcinomas a substance which could be the same material as that seen by us *Santesson* (28) called attention to its more frequent occurence in preoperatively irradiated tumours We detected this substance in 13 out of 17 irradiated and in 7 out of 17 non irradiated cases of mammary cancer, all treated in the Radiumhemmet.

In our cases previously seen a positive amyloid staining with Congo-red occurred, even responsiveness to methyl violet, iodine-green and Lugol's solution might often occur It was remarkable that the amyloid positive fibrills as well as the amorphous substance picked up elastica stains, although the "classic" amyloid never react with them

Civatte (4), *Ganter and Beurlet* (9) reported the findings of amyloid deposition in basal cell carcinoma Although the amyloidotic nature of the

Received 6 viii 71

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substance observed by these authors was not definitively proved due to lack of polarisation studies, it seems likely that their findings correspond to our present description of an amyloid like material

Due to differences between common amyloid and the material here described, the term *"elastic amyloid'* was proposed Fibrinoid necrosis could be excluded

The aim of the present study was to evaluate previous observations on a broader basis and to contribute to the knowledge of their significance

MATERIAL AND METHODS

Twenty indifferentiated, fibrous, non irradiated mammary carcinomas were collected from surgical material Forty basal cell carcinomas were investigated, they were all excised for diagnostic purposes The specimens were stained with

a haematoxylin eosin resp Weigert-van Gieson s stain
b Elastica stain Resorcin fuchsin + polychrome methylenblue
c Amyloid stains Congo red, a m Roulet, methylviolet, iodine green, malachite green, Lugol's solution
d Polychrome methylenblue with Toluidine blue
e PAS reaction
f Alcian blue
g Mallory's phosphotungstic haematoxylin as modified by Ladewig
In addition the following methods were used
h Fluorescence microscopy, Wild-Heerbrugg microscope, 1, using acridine orange as fluorochrome 2, fluorescence after Congo red staining
i Dichroic staining of slices stained with Congo-red in polarized light (17, 21, 27)

RESULTS

I *Mammary Carcinoma*

In *haematoxylin eosin* preparations a peculiar substance bound to loose fibres could be observed between the cancer cells Some cancer cell nests were surrounded by such fibres, which stained red to a significantly higher degree than other tissue components In other areas both the tumour cells and the fibres showed regressive changes Fibrils split in the same manner that described by *Pearse* (25) in the case of ageing elastic tissue and



Fig 1 Elastic amyloid mass within mammary carcinoma Necrobiotic tumour cells in the centre Weigert van Giesons stain, × 400

occasionally a granulated respectively homogenous material emerged while produced an eosinophilic layer around cancerous foci Tumour cells displayed necrobiosis as well as necrosis and finally cells might be completely absent or only some centrally located residual elements might remain preserved *Weigert van Giesons* stain produced the foreign substance in a bright yellow colour and the yellow fibres could be easily discriminated from the red connective tissue The granular or homogenous material was also stained yellow (Fig 1) The fibres and the granular resp homogenous substance reacted intensively with *elastica stains* The homogenous degeneration of elastic fibres suggested staining with *amyloid dyes* Indeed staining with Congo red proved to be the adequate method The substance sticking to elastic fibrils around the cancerous foci showed an intensive brownish red hue, this colour, however, was somewhat more brownish and less red than that of common amyloid (Fig 2) Other amyloid stain-



Fig 2 Detail of the previous figure Positively stained with Congo red × 200

ing methods especially treatment with methyl violet also reacted more or less positively *In fluorescence microscopic studies* a clear yellowish silver green fluorescence of the massiv foci was seen This colour was less intense than that of common amyloid the involved elastic fibrils however showed bright fluorescence identical to that of primary or secondary amyloid

Divry and Florkin (6) *Romhanyi (27) Ladewig* (17) *Lutly (21)* observed a birefringence characteristic of amyloid stained with Congo red *Missmahl* (24) emphasized the briefringent substances green colour *Our polarisation microscopic study* showed no such reactivity of elastic amyloid

In order to explore whether this variant of amyloid contains carbohydrates or glycoproteins further histological studies were undertaken In several cases a weakly positive *PAS reaction* was noted If *Alcian blue* were used minimal greyish blue tinged deposits would be observed in the substance The periphery of cancerous foci—probably corresponding to areas where the new substances were continously deposited and adsorbed by the fibres – if *Ladewigs* method were used would produce an intense red colour whereas the central parts remained unstained Thus according to these histochemical investigations confirmed by experimental studies by *Christensen* (5) and others elastic amyloid contains acid—and neutral mucopolysaccharides and glycoproteins in addition to proteinic substances

II *Basal Cell Carcinoma*

Studies on basal cell carcinoma showed the development of elastic amyloid in its earliest stages In 50 % of our cases small homogenized or somewhat fibrillary intercellular deposits occurred in the centres of the solid tumour nests The deposits stained orange yellow in *Weigert van Gieson* sections and displayed a positive *PAS reaction* (Fig 3) The substance was stained sky blue with *Alcian blue* and a fine metachromasia appeared after *toluidine blue* staining On the other hand it remained refractory against staining with *elastica dyes* and *Congo red* The metachromatic substance was observed even in some tumour cells The substance thus showing staining properties identical to those of precursors of experimentally produced amyloid (5) subsequently spreads to the periphery of the tumour nests where ist adsorption to proliferating elastic fibrils could be easily followed If adsorbed to fibrils the morbid material at that site became responsive to *Congo red* as well at to *elastica stains* and appeared bright red if treated by *Ladewigs method* (Fig 4) The positive PAS reaction and toluidine metachromasia gradually disappeared In contrast to findings in cases of mammary cancer the substance which was also present in spaces between tumour cell nests showed only a mild degree of granular condensation and homogenisation (Fig 5) After treatment with *acridin orange* a bright fluorescence of the Congo positive elastic fibrils and rather weak fluorescence of the homogenized parts arose Whereas the initial deposits in the tumour

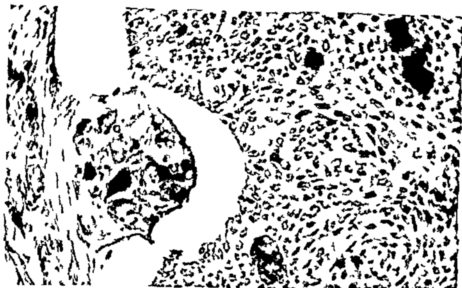


Fig 3 Globuli in basal cell carcinoma and in surrounding connective tissue PAS reaction × 400

nests did not show any *fluorescence after Congo-red staining*, a red fluorescence of the newly developed elastic fibres became evident in older foci and in the homogenized parts The same fluorescence could be observed in the Congo positive parts of the mammary cancers *Birefringence* of Congo-red stained specimens could not be observed

III *Control material*

Senile elastosis, often occurring in areas around basal cell carcinoma, showed intensive elastica staining, displayed a yellowish colour in Weigert-van Gieson sections and a light brownish colour after Congo red treatment No fluorescence, either after acridine orange or Congo-red staining could be observed, other methods by which to detect amyloid gave also negative results

Material from cases of *primary and secondary amyloidosis* and *amyloidosis of the skin* showed histologically positive amyloid reactions and as already mentioned, a clear yellowish green fluorescence in preparations treated with acridine-orange and a red fluorescence after staining with Congo-red Furthermore a greenish or yellowish birfringence was seen in specimens stained with Congo-red Such amyloid deposits, however, were never reactive to elastica stains, to Ladewig's method, or to Alcian blue, only the PAS reaction was occasionally weakly positive



Fig 4 Increase of amyloid impregnated elastic fibres in basal cell carcinoma stained red with Ladewig's method in the figure black × 400

Fibrinoid necrosis developed in the renal vessels of a patient affected by systemic lupus erythematosus and in the tonsillary arteries of a patient with periarteritis nodosa Elastica and amyloid stainings were negative, whereas the PAS reaction was positive and Ladewig's method produced a red stain of the diseased vessel walls A light olive green fluorescence appeared in the kidney after acridine orange staining The results are summarized in Table 1

DISCUSSION

A peculiar, fibrillary, granular or amorphous substance was found to be associated with nests of basal cell carcinomas The rate of occurrence of the same material was found to be relatively high in cases of fibrous mammary carcinomas, beeing lower in certain carcinomas of the recto sigmoid, urinary bladder or parotid gland This substance showed certain morphological and histochemical reactions typical for amyloid and furthermore presented important discriminative characteristics It reacted with elastica dyes and displayed *no birefringence after Congo-red* staining as otherwise seen in the cases of common amyloidosis Differences could also be observed with the fluorescence microscope It was *Ph Schwartz* who pointed out the yellowish fluorescence in certain parts of the aorta, pancreas, myocardium, brain and coronary vessels of elderly individuals, or aged dogs, using Thioflavine S as fluorochrome (32, 33, 34, 35) Such fluorescent deposits displayed positive amyloid staining Such intensive, yellowish green fluorescence of the amyloid like material was also observed in our study, although a different fluorochrom was used This fluorescence was less intense than the luminescence of the deposits in cases of common amyloidosis or cutaneous amyloidosis. Thus, the term "elastic amyloid" is recommended to apply for the bescrived substance

The possible coexistence of basal cell carcinoma and senile elastosis as well as the need for a differentiation between these two



Fig 5 Elastic amyloid within basal cell cancer Weigert - van Gieson stain × 200

conditions should be emphasized Treatment with toluidine blue of senile elastosis, however, produces no metachromasia, no positive PAS-reaction, and no staining with Alcian blue or staining according to Ladewig's method could be observed

Under the influence of studies by *Letterer* and his school (18, 19, 20), the importance of immunobiological implications in the pathogenesis of amyloidosis has been widely assumed Amyloid seems to result from one abnormal immune mechanism and may represent the outcome of a precipitation process (3, 19, 20, 35)

According to *Teilum* (36, 37, 38) the appearance of a peculiar, homogeneous, hyalin like eosinophilic substance could be considered the morphological manifestation of an antigen antibody reaction (14, 29) This substance easily joints with various fibrillary and membranous elements (10 12) and, [illegible] develop amyloidosis, a process, which is enhanced by the application of nitrogen mustard or cortisone (5)

TABLE 1 Results of the Described Histological and Histochemical Investigations on Common – and Elastic Amyloid and Control Material

Stains/methods	Reactivity of the common amyloid	elastic* amyloid	senile elastosis	fibrinoid necrosis	Remarks on elastic amyloidosis*
Haematoxylin eosin	intensive red	intensive red	pale red	intensive red	only in mammary cancer
Haematoxylin – van Gieson	yellow	yellow	yellow	yellow	
Elastica staining with recorsin fuchsin	—	+ + +	+ + +	—	
Congo red	+ + + red	+ + + brownish red	—	—	
Methyl violet	+ + +	+	—	—	not always stainable
Toluidine blue	—	+ +	—	—	only in early stages of basal cell cancer
PAS	— or +	I —, or + II + + +	—	+ + +	only in early stages of basal cell cancer
Alcian blue	—	I — II + + +	—	—	only in early stages of basal cell cancer
Mallory's trichrome, modified by Ladewig	—	I — II + + +	—	+ + +	only in early stages of basal cell cancer
Polarization microscopy after Congo red	+ + +	—	—	—	
Fluorescence michoscopy after acridine orange	+ + + greyish yellow	+ + greenish yellow	—, or +	seldom + olive green	
Fluorescence microscopy after Congo red	+ + + red	+ + + red	—	—	

* I Mammary cancer II Basal cell carcinoma

According to *Christensen* (5) an experimental PAS positive pre amyloid, maturing *in situ*, later on becomes surrounded by Alcian positive substance Thus, the presence of acid mucopolysaccharides, neutral polysacc-harides and glycoproteides may be assumed during the development of experimental amyloid deposits The pre-amyloiditic substance proved to be *Congo negative* in this phase! The initial tinctorial properties decreased later on and the material could then be stained with Congo red

Similarly, in the present study, basal cell cancer nests with PAS- and Alcian blue positive foci were seen, which could be stained also metachromatically by toluidine blue The intensity of these stainings decreased, while the Congo red reaction became increasingly accentuated At the same time and parallel with this changes, elastica stainings became positive and fluorescence appeared Birefringence, as seen in spontaneous or experimental amyloidosis was never observed The tinctorial changes of the amyloid substances described in this study are possibly due to alterations in the chemical composition, in which the time factor and different maturation phases play an eminent role

It is known that proteins in the body may become antigens when modified under pathological conditions and that the response of the organism is a production of antibodies Much work has been done to prove the presence of specific antigens in experimental, chemical, and viral tumours The existence of such antigens was verifyed by *fluorescence*

techniques and their presence seems to be probable even in certain human neoplasms such as Burkitt's lymphoma and nasopharyngeal cancer (15, 16) According to the above mentioned *observations* the appearance of the elastic amyloid may be related to immunological processes arising around nests of mammary- and basal cell carcinoma In our opinion the deposition of elastic amyloid could be one of the morphological aspects of an antigen antibody reaction, it may be a manifestation of defensive mechanisms in the cancerous organism. The elastic amyloid *represents* a phase *of maturation in the deve*lopment of amyloid Whether it can be transformed into common amyloid cannot be decided, for the time being Further work on this question is in progress using other methods, for instance experiments with elastase digestion

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This study has been supported by the *Edward Welanders Stiftelse*

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Acta path microbiol scand Section A 80, 193–200, 1972

STUDIES OF DIABETIC GLOMERULOSCLEROSIS USING AN IMMUNOFLUORESCENT TECHNIQUE

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Kidney Tissue from 22 patients with diabetes mellitus was examined using an immunofluorescent technique The presence of the following substances was looked for gamma G-globulin, complement, beta lipoprotein, fibrinogen, albumin, insulin and anti insulin Diffuse and nodular lesions were found in some, but not all cases, to contain slight amounts of gamma G globulin, and at times albumin Fibrinoid caps and hyalinized arterioles revealed the presence of complement, beta lipoprotein, fibrinogen and occasionally gamma G globulin, *but not albumin There was no evidence for the presence of insulin or anti insulin* in the kidney lesions The results are discussed They do not seem to support the theory of an immunologic pathogenesis of diabetic nephropathy

The pathogenesis of the characteristic lesions of the diabetic kidney—the diffuse and the nodular forms of glomerulosclerosis and the exudative lesions (fibrinoid caps, capsular drops and arteriolar hyalinosis)—is still unsettled and disputed

Knowledge of the chemical composition of these different lesions is a prerequisite for discussion of pathogenesis

The immunofluorescence technique provides in theory a well suited histochemical method for localization of proteins in histological structures, and this technique has been used to study diabetic kidney tissue

However, the relatively few comprehensive investigations of this kind have given such diverging results that a definitive determination of proteins in the lesions of diabetic kidney cannot be said to have been performed Presumably, this may be due to differences in technique which, moreover, often is difficult and erratic

Because of the rather strongly diverging results, we have considered it to be of interest, once again, to use the immunofluorescence technique in order to determine whether certain proteins may occur in diabetic kidney lesions

MATERIALS AND METHODS

The material investigated consisted of human kidney tissue from 22 patients with diabetes mellitus of various durations and severities

Table 1 gives a survey of the material and a semiquantitative estimation of the severity of the kidney lesions involved

Of the 22 specimens of diabetic kidney tissue, 17 were taken at autopsy, five at biopsy

Handling of the Tissue

a) autopsy tissue Blocks of unfixed tissue about 3 mm in thickness were quick frozen at − 70° C in isopentane in a container with a dry-ice acetone freezing mixture The tissue blocks were stored at − 70° C until required Other blocks of tissue were fixed in formalin and embedded in paraffin,

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sectioned and then stained with haematoxylin eosin and periodic acid Schiff b) biopsy tissue Tissue was taken by needle biopsy A part of it was quick frozen in a matrix of gelatine at —70° C and stored at this temperature Another part was fixed in Carnoy's fluid and embedded in paraffin for preparation of conventional sections

The frozen blocks of tissue were sectioned in a cryostat at —20° C Six micron thick consecutive sections were cut and placed on clean slides where they were allowed to dry for about 30 minutes at room temperature

Antisera

For the investigation of gamma G globulin, complement, beta lipoprotein, fibrinogen and albumin, the following commercially purchased antisera were used as the *first* layer in the indirect immunofluorescence method

1) rabbit anti human gamma G globulin (Beringwerke)
2) rabbit anti human complement (Beringwerke)
3) rabbit anti human beta lipoprotein (Hyland)
4) rabbit anti human fibrinogen (Nordic Diagnostics)
5) rabbit anti human albumin (Nordic Diagnostics)

As the *second* layer was used

1) FITC labelled goat anti rabbit gamma globulin (Nordic Diagnostics)

For the examination of insulin by the indirect immunofluorescence method the following antisera were used

As the *first* layer non labelled guinea pig anti bovine insuline serum (Hyland)

As the *second* layer FITC labelled rabbit anti guinea pig gamma globulin (Nordic Diagnostics)

Staining

Before adding the different antisera slides were washed in phosphate buffered saline (pH 7 1) for 3×5 minutes Then the first layer was applied for 30 minut
3×5 mir
minutes
saline for 3×5 minutes They were then dried for about 10 minutes at room temperature and mounted in Fluomount

Examination

After staining, the slides were examined in a Zeiss Universal Fluorescence Microscope with a Tiyoda condensor and Osram HB 200 mercury lamp as the source of light The primary filters were BG 38 and BG 12 or an interference filter especially adapted for fluorescein isothiocyan (Rygaard and Olsen 1969) Secondary filters c sisted of type no 44 or no 50 Kodak High Spe Ectachrome film was used On the microsco examination we have attempted, as far as possib to observe and photograph exactly the same stru tures, as they occurred in the sequential sectio stained for different proteins Thereby a go comparison between the single stainings is achiev and between these and the controls

Controls

a) *Technical controls* For control sections wi the indirect method were used

1) sections where the first layer was norm rabbit serum, instead of a specific rabb antiserum (in the examination for insuli normal guinea pig serum) The second lay was as described
2) sections on which only the FITC labelle second layer was applied
3) unstained sections

Every fluorescent structure was compared wit exactly the same structure in sequential section. Only if fluorescence in these was totally absent, o at least definitely weaker than the fluorescence o the sections stained with antiserum we considere the fluorescence to be specific i e an expression of a reaction between antibody and antigen (fig 5 and fig 6)

b) *Patient controls* In order to control the method, kidney tissue from 16 non diabetic patients without clinical evidence of kidney disease and without macroscopic kidney lesions was studied The non diabetic patients were about the same age as the diabetic patients Fourteen of these patients revealed arterioles with various degrees of hyalinization In addition, kidney specimens from patients with Alport s syndrome (1) malignant hypertension (1), membraneous glomerulonephritis (1) and amyloidosis (1) were examined

In order to control the applicability of anti insulin in the indirect immunofluorescent technique the staining procedure was performed on fresh, quick frozen pancreatic tissue from young rats This resulted in a strong fluorescence in the beta-cells of the islets as an expression of the binding of the anti insulin to the insulin in the beta cells, whereas fluorescence was not found in the control sections

FITC labelling of Insulin

In order to assay the content of anti insulin in the diabetic kidney tissue FITC labelled insulin was produced in the following way To 10 ml of insulin (Actrapid Novo) 1 ml 0 5 M sodium bicarbonate buffer (pH 9 0) and then 0 75 mg fluo-

rescein isothiocyanate was added After stirring at 4° C for 24 hours the solution was 1) dialysed *against Macrodex for 18 hours 2) rapidly* passed through a column of G25 Sephadex, which was equilibrated in 0 05 M sodium phosphate buffer (pH 7 5) containing a solution of NaCl at 0 9 per cent.

The FITC labelled insulin was used in the direct method After washing in buffered saline it was applied for 30 minutes, the slides were then washed again in buffered saline for 3×5 minutes, dried and mounted as previously described

RESULTS

A. *The Diabetic Material*

Ordinary Light Microscopy

In all kidney specimens from the 22 diabetic patients, diffuse, PAS positive glomerulosclerosis was seen The degree of alteration varied from quite slight to very severe In 12 kidneys nodules were present in the glomeruli

Six kidneys contained fibrinoid caps in the glomeruli, and in six kidneys scattered capsular drops were present

In all 22 kidneys hyalinization and thickening *of the walls of preglomerular arterioles* was seen Here, too, great variation between cases was seen

The results are summarized in *Table 1*

Examination of FITC-stained Sections

The diffuse lesion Gamma G globulin was found in 13 diffuse lesions, however, in most cases to only a slight degree In typical cases fluorescence was seen in the thickened capillary walls of the glomeruli, but never as strong as can be seen in other kidney dis

TABLE 1 *Semiquantitative Evaluation of the Degree of Diabetic Glomerular Lesions (—, +, + +, + + +) in Slides Stained with Periodic acid Schiff*
(Case 13 16 17, 18 and 20 were studied using biopsy material, all others with autopsy material)

case number	sex	age	age at beginning of d m	totally sclerosed glomeruli	diffuse lesion	nodular lesion	fibrinoid caps	capsular drops	arteriolar hyalinosis
1	f	41	22	+ + +	+ + +	+ + +	+ + +	—	+ +
2	f	38		+	+	—	—	—	+
3	m	44		+ + +	+ + +	+ + +	+ + +	—	+ + +
4	m	50		+	+ +	+	—	—	+ +
5	m	48	31	+	+ + +	+ +	—	+	+ + +
6	f	59	40	+ +	+ +	+ +	—	+	+ +
7	f	70	50	+	+ + +	+ + +	—	+	+ + +
8	f	81	69	+	+	—	—	—	+ +
9	m.	45	23	+ + +	+ + +	+ + +	+ +	—	+ +
10	f	73	50	+ +	+ + +	+ + +	+ +	+	+ + +
11	m	73	64	+	+ +	—	—	—	+ + +
12	m	68	50	+	+ +	+	—	—	+ +
13	m	37	22	—	+	—	—	—	+
14	m	67	34	+	+	—	—	—	+ +
15	f	48	37	+ +	+ +	—	—	+	+ + +
16	m	23	6	—	+ + +	+	—	—	+ + +
17	f	65	51	—	+ + +	+	—	—	+ + +
18	m	24	15	—	+	—	—	—	+
19	f	74	70	—	+ +	—	+	—	+ +
20	m	43		—	+ + +	+ +	—	—	+ + +
21	f	65		+	+ +		—	—	+ +
22	f	40	10	+ + +	+ + +	—	+ + +	+	+ + +

13*

TABLE 2 *Localization of Plasma Proteins in Kidney Tissue from 22 Patients with Diabetes Mellitus*

	diffuse lesion	nodular lesion	fibrinoid caps	hyalinized arterioles	Bowmann basement membrane	tubular basement membrane
gamma G globulin	+ 4/22 (+) 9/22 — 9/22	+ 3/12 (+) 6/12 — 3/12	+ 4/6 (+) 1/6 — 1/6	+ 10/22 (+) 3/22 — 9/22	+ 4/22 (+) 5/22 — 13/22	+ 3/22 (+) 5/22 — 14/22
complement	+ 0 (+) 0 — 22/22	+ 0 (+) 0 — 12/12	+ 3/6 (+) 2/6 — 1/6	+ 12/22 (+) 8/22 — 2/22	+ 0 (+) 1/22 — 21/22	+ 0 (+) 0 — 22/22
beta-lipo protein	+ 0 (+) 2/19 — 17/19	+ 0 (+) 0 — 12/12	+ 6/6 (+) 0 — 0	+ 16/19 (+) 2/19 — 1/19	+ 0 (+) 0 — 19/19	+ 0 (+) 0 — 19/19
fibrinogen	+ 0 (+) 3/21 — 18/21	+ 0 (+) 0 — 12/12	+ 6/6 (+) 0 — 0	+ 7/21 (+) 10/21 — 4/21	+ 0 (+) 0 — 21/21	+ 0 (+) 0 — 21/21
albumin	+ 0 (+) 5/21 — 16/21	+ 1/12 (+) 3/12 — 8/12	+ 0 (+) 0 — 6/6	+ 0 (+) 0 — 21/21	+ 4/21 (+) 8/21 — 9/21	+ 5/21 (+) 8/21 — 8/21

\+ = positive (+) = slightly positive — = negative

Number over fraction line number of cases with the protein concerned
Number under fraction line number of cases with the lesion concerned

orders, for example membraneous glomerulo nephritis or lupus nephritis (Fig 2)

In the mildest forms of the diffuse lesions gamma G globulin was not demonstrated

A slight amount of *albumin* was found in five fully developed cases of the diffuse lesions Diffuse extensions of *Beta lipoprotein* and *fibrinogen* were demonstrated in a few glomeruli of two and three patients, respectively

The nodular lesion Gamma G globulin was found in 9 out of 12 cases with nodular lesions The positive findings occurred where the glomeruli contained fully developed noduli, and in addition were relatively well preserved (fig 1) Glomeruli with more severe alterations and more irregular nodular formations did not reveal the presence of gamma G globulin

Albumin was found in noduli in four cases, but fluorescence was somewhat weaker

Complement, beta-lipoprotein and *fibrinogen* were not demonstrated in the nodular lesions

Fibrinoid caps Among the six cases where fibrinoid caps were present five had also severe lesions of the other types

Gamma G globulin and *complement* were found in five, *beta lipoprotein* and *fibrinogen* in all six cases *Albumin* was not demonstrated

The exudations were often large and amorphous and fluorescence was usually stronger than in the diffuse and most cases of the nodular lesions (fig 3)

Capsular drops This lesion was found in some of the paraffin sections but was very difficult to preserve in frozen sections and consequently very few were encountered This lesion seems to contain a slight amount of gamma G globulin

Hyalinized arterioles Juxtaglomerular arterioles with hyaline changes of varying degree were found in all 22 kidneys.

Gamma G globulin was often, but not constantly, localized to the hyalinized, thickened walls of arterioles *Complement, beta lipoprotein* and *fibrinogen* were found in most of the juxtaglomerular, hyalinized arterioles, this was especially the case with lipoprotein, which as a rule yielded the most brilliant fluorescence (fig 4) The four proteins were often, but not nearly always, encountered in the same arterioles Fluorescence *in the arterioles appeared similar fluorescence* in the fibrinoid caps

Albumin was not demonstrated in hyalinized arterioles

Other structures

In the basement membrane of the Bowmann capsule and the proximal tubules, a *moderate fluorescence corresponding to a* content of *gamma G globulin* and *albumin* was seen in many cases, whereas the other proteins were not demonstrated here

The results are summarized in *Table 2*

Insulin and anti insulin Examination for these substances using guinea pig *anti insulin* and FITC labelled insulin respectively was performed in 10 of the 22 specimens of diabetic kidney tissue Specific fluorescence was not seen in any of these

B *The Non Diabetic Material*

On immunofluorescent examination of kidney specimens from the 16 patients without clinical kidney disease, a slightly positive reaction for gamma G globulin was encountered in hyalinized arterioles *in two* cases At the same site, a positive reaction for complement was seen in eight cases, for beta lipoprotein in 11 cases, and fibrinogen in six cases Albumin was not demonstrated in arterioles

There was no reaction in the glomeruli of these patients with the exception of two cases which revealed a slight content of either albumin or gamma G globulin

The patient with *membraneous glomerulonephritis* revealed a positive reaction for gamma G-globulin and complement in the glomeruli, and to a lesser degree, fibrinogen In the kidney tissue from the patient with *malignant hypertension a strong reaction for* fibrinogen was seen in the glomeruli and arterioles Beta lipoprotein was also found in these structures Gamma G globulin was seen in the glomeruli, but not in arterioles In the glomeruli and arterioles from the patient with *Alport s syndrome,* beta lipoprotein and fibrinogen were demonstrated The patient with *amyloidosis* revealed a strong reaction for gamma G-globulin and fibrinogen in the glomeruli and arterioles

DISCUSSION

In *Table 3* the most important results obtained in this study are compared with corresponding results from the most comprehensive of the previous immunofluorescent investigations of diabetic kidney tissue

As will be seen from Table three, the divergencies between the previous examinations are considerable, and our results too *differ from those of the previous examinations*

The results of this study agree best with those of *Burkholder* (1965) and deviate only slightly from his results We have not been able to confirm *Burkholder's* finding of only sparse insulin mesangially in the glomeruli, conversely, we have demonstrated albumin in the glomerular noduli of a few patients, and fibrinogen in the exudative lesions

Our results coincide with those of *Davies et al* (1966) concerning the demonstration of beta lipoprotein and fibrinogen in the exudative lesions, but we cannot confirm these investigators negative findings of gamma G globulin in glomeruli

As *Berns' et al* (1962), we found gamma G globulin in glomeruli and in hyalinized arterioles

Regarding the demonstration of the possible presence of insulin and anti insulin *in certain diabetic lesions, as mentioned* previously, we have not found evidence of a presence of these substances This is in dis*agreement with Berns' et al* (1962), who observed fluorescence, indicating that these

TABLE 3 *Comparison between Previous and own Results of Immunofluorescent Examination of Diabetic Kidney Tissue*

	protein demonstrated	Berns et al 1962	Burkholder 1965	Farrant & Shedden 1965	Davies et al 1966	Frokjær Thomsen 1971
diffuse lesions	γ G globulin		+ 6/6		— 0/13	+ 13/22
	Complement		+ 5/5*			— 0/22
	beta lipoprotein				+ 13/13	+ 2/19
	fibrinogen		+ 2/5		+ 13/13	+ 3/21
	albumin		+ 3/5		— 0/13	+ 5/21
	insulin		+ 4/5**			— 0/10
	anti insulin			+ 28/32		— 0/10
nodular lesion	γ G globulin	+ 12/25	+ 4/4		— 0/13	+ 9/12
	Complement		— 0/4			— 0/12
	beta-lipoprotein				+ 13/13	— 0/12
	fibrinogen	— —/—	— 0/3		+ 13/13	— 0/12
	albumin	— —/—	— 0/3		— 0/13	+ 4/12
	insulin	+ 2/2	— 0/6			— 0/12
	anti insulin	+ 24/25		+ 28/32		— 0/10
fibr caps + caps drops	γ G globulin		+ 5/5		— 0/13	+ 5/6
	complement		+ 5/5			+ 5/6
	beta lipoprotein				+ 13/13	+ 6/6
	fibrinogen		— 0/4		+ 13/13	+ 6/6
	albumin		— 0/4		— 0/13	— 0/6
	insulin		— 0/6			— 0/6
	anti insulin					— 0/6
hyalinized arteriol	γ G globulin	+ —/25	+ 5/6		— 0/13	+ 13/22
	complement		+ 5/6			+ 20/22
	beta lipoprotein				+ 13/13	+ 18/19
	fibrinogen		+ 1/5		+ 13/13	+ 17/21
	albumin		— 0/5		— 0/13	— 0/21
	insulin	+ 2/2	— 0/6			— 0/10
	anti insulin	+ 24/25		+ —/32		— 0/10

Over fraction line = number of positive
Under fraction line — number of examined cases

* Focally in a few glomeruli
** Small slightly positive mesangial foci

ubstances were located in nodular lesions and in hyalinized arterioles On the basis of this observation the theory was proposed that these lesions might contain complexes of insulin and anti insulin and that an immunologic reaction was an important component in the pathogenesis of diabetic kidney lesions The insulinbinding properties of the kidney lesions were supposed to develop as a consequence of treatment with heterologous insulin and the resultant production of anti insulin

Farrant & Shedden (1965) using FITC labelled insulin demonstrated anti insulin diffusedly and nodularly in glomeruli and hyalinized arterioles in a number of diabetic patients However these investigators doubted whether this binding was immunologically specific, as the FITC labelled insulin also bound well to kidney tissue in diabetics that were not treated with insulin and indeed in some non diabetic patients

It should be mentioned that *Berns et al* and *Farrant & Shedden* used formalin fixed paraffin embedded tissue in their investigations a technique which we have attempted but failed to achieve reliable results with, on account of a marked tendency to autofluorescence As previously mentioned *Burkholder* (1965) using FITC labelled anti insulin

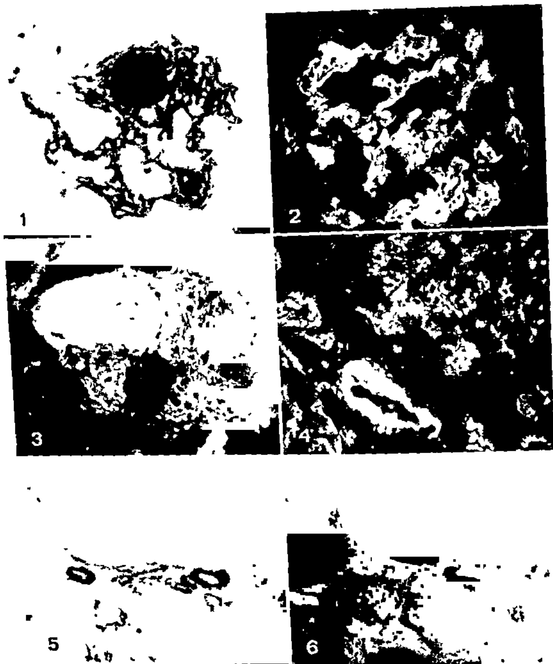


Fig 1 Glomerulus with fluorescent noduli in section stained for gamma G globulin × 275

Fig 2 Glomerulus with fluorescence in the diffuse lesion in section stained for gamma G globulin × 275

Fig 3 Glomerulus with a large, fluorescent exudative lesion ("fibrinoid cap") in section stained for lipoprotein × 275

Fig 4 Fluorescent hyalinized juxtaglomerular arteriole in section stained for lipoprotein × 275

Fig 5 Two fluorescent, somewhat hyalinized, arterioles in section stained for complement. × 275

Fig 6 Same motive as in Fig 5 in a consecutive section, stained with a normal rabbit serum, instead of a specific antiserum Absence of fluorescence in the arterioles serves as a control. × 275

applied to frozen sections, was able to trace small fluorescent foci mesangially in glomeruli, indicating the presence of insulin, whereas insulin was not found in the noduli or hyalinized arterioles

On the basis of the present and earlier investigations, we consider it fairly certain that some, but not all of the diffuse and nodular lesions contain small amounts of gamma G globulin and that fibrinoid caps and hyalinized arterioles contain complement, beta lipoprotein and fibrinogen and often gamma G globulin as well

Staining for insulin and anti insulin did not yield evidence of a presence of these substances in the diabetic kidney lesions, in particular this investigation does not support the theory that complexes of insulin and anti insulin are involved in glomerular and vascular lesions

Regarding the interpretation of the various results, some caution should be exercised The immunofluorescence method may, with correct technique, yield information as to the localization of a substance in relation to histological structures However, such localization—for example of gamma G globulin—does not prove that an immunologic reaction has resulted in deposition of the substance in question Under certain circumstances the findings may *suggest* an immunologic pathogenesis for example the prescence of gamma G globulin *and* complement in the same histological structure or lesion (*Burkholder* 1965 *Freedman* & *Markowitz* 1962)

The presence of gamma G globulin in some—but not all—of the diffuse and nodular lesions of the glomeruli and the fact that albumin was found in some of these lesions do not suggest an immunologic pathogenesis of the glomerular lesions

The presence of gamma G globulin and complement in the same lesions—encountered in most of the fibrinoid caps and in many hyalinized arterioles—might, in connection with the negative finding of albumin in these structures support, but not prove the theory of an immunologic pathogenesis of these lesions However, the examination very clearly shows that beta lipoprotein and fibrinogen are also present in the exudative lesions, this is supported by the data advanced by *Davies et al* which are very clear in this respect

Thus, even if the simultaneous presence of gamma G globulin and complement might in itself suggest an immunologic pathogenesis of the exudative lesions the presence of beta lipoprotein and fibrinogen might rather lead to the interpretation that an exudation of several plasma proteins takes place this process might well be selective as albumin is apparently not found in the exudative lesions

Consequently, our results do not seem to support the theory of an immunologic pathogenesis of diabetic nephropathy

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I thank Dr H Ørskov 2nd medical department Kommunehospitalet Arhus for technical assistance in preparing the FITC labelled insulin.

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THREE-DIMENSIONAL RECONSTRUCTION OF INTRAHEPATIC BILE DUCTS IN A CASE OF POLYCYSTIC DISEASE OF THE LIVER IN AN INFANT

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The anatomy of the intrahepatic biliary system was evaluated by means of three dimensional reconstruction of a portal tract in the liver from a stillborn infant with polycystic disease of

In a previous paper (3) a report was made of the peculiar arrangement of the bile ducts in the portal tracts of a liver which in all probability should be classified as congenital hepatic fibrosis. Three dimensional reconstruction revealed that the bile ducts in part had the form of curving plates. A hypothesis of the pathogenesis of the disorder was proposed based on the embryology of the bile duct system. The relation to the more common hepatic malformation polycystic disease of the liver was discussed. If a similar malformation of the intrahepatic bile duct system could be proven in this last condition, it would constitute strong evidence for the close relationship between the two congenital hepatic disorders and a possible common pathogenical pathway. This paper reports the study of the liver from a stillborn infant with polycystic disease of the liver.

Received 23.xii.71

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CASE REPORT

A healthy woman, aged 20 years, was admitted to hospital for delivery 6 weeks before term. The pregnancy, which was her first, had been normal with no family history of congenital malformations. No liver or renal disease in the family was recorded. The labour was difficult and resulted in a stillborn girl, weight 3400 g and length 47 cm. Autopsy was performed and the main diagnoses were a large occipital meningocele and polycystic disease of the liver and kidneys.

Spleen, *pancreas* and *lungs* were found normal without cysts.

The *kidneys* were both greatly enlarged, measuring 13 × 7 × 6 cm, weight 245 g (normal weight about 25 g). They were completely transformed to a small cystic, sponge like tissue. At microscopy the cysts lined with a single row of cuboidale cells were embedded in a loose connective tissue with scattered immature glomeruli in different stages of development and foci of haematopoietic cells. The renal pelvis and ureter were normal.

The *liver* (Fig. 1) measured 15 × 8 × 4 cm, weight 210 g (normal about 110 g). The surface was smooth except for a few small cysts in the right lobe, but the cut surface revealed a multitude of cysts ranging from 1 mm to 3 cm and containing a light green viscous fluid. The parenchyma between the cysts looked normal. The gall bladder and external bile ducts were normal. Histologically the



Fig 1 Macroscopic picture of the liver The largest cysts were situated in the centre of the liver and are not visible

cysts were lined with a single layer of cuboidal or flattened epithelium and apart from the distorsion of the parenchyme caused by these cysts the basic lobular pattern was intact There was a moderate periportal fibrosis consisting of mature collagen, but without inflammatory exudation The most conspicuous feature was an excess of bile ducts in the connective tissue They were to a very high degree cut longitudinally (Fig 2) and often displayed a circular course around vessels or around a core of connective tissue (Fig 3) The epithelium lining these ducts was cuboidal or low columnar in most areas of normal appearance, in places degenerated No mitoses were seen In some places the epithelium only consisted of one row of cells The parenchymal cells looked normal and were arranged in a muralium simplex or duplex No bile stasis, necrosis or cellular infiltrates were seen except foci of haematopoietic cells The vessels seemed normal

The portal space in Fig 4 was chosen for three dimensional reconstruction following the technique previously described (2)

The magnification of the microscope plus drawing device is 185 × The reconstructed block measured 13 × 8 5 × 6 cm (Fig 5) Colours facilitate very much the evaluation of the reconstructed model which may be difficult in a black and white picture A schematic drawing of the reconstructed structures is therefore given in Fig 6

The result was that to be expected from appliance of stereometrical principles on these longitudinally cut ducts As seen in Figs 5 & 6 the duct system in part has the form of a curved plate which gradually breaks up and ends in tubular ducts of usual dimensions The ductal plate has many connections to the limiting plate bordering the portal tract Many of the tubular ducts originating from the ductal plate are interrupted and some are blindly ending The portal vein branch in the reconstruction seems to be normal

DISCUSSION

Much speculation regarding the aetiology and pathogenesis of polycystic disease of the liver has been present in a great number of papers Several reviews of the literature exist e g in the paper of *Norris and Tyson* (8)

The congenital character of the disease and the close relation to polycystic disease of the kidneys is generally agreed, although both malformations may appear independent of the other (*Melnick* (5) and *Rall & Odel* (9)) In addition to sporadic cases of polycystic disease of the liver, a heredo familial tendency is evident

Concerning the pathogenesis, the ductal origin of the cysts has not been challenged since the descriptions by *Moschcowitz* (7) and *von Meyenburg* (6) appeared, but the problem has been to explain the epithelial or ductal source of the cysts and the mechanism in their development

The cysts – being the most conspicuous feature of the disease – may be present in very variable numbers and sizes Cysts visible to the naked eye may be found at birth as in this case, but seems rarely encountered, and it is obvious that cystic dilatation and enlargement may take place later in life Apart from the cysts all histological descriptions include 'excessive numbers of bile ducts', believed to be the source of the cysts The origin of these bile ducts has been a matter of dispute The denomination 'aberrant' is fitting for the histological appearance and it is less probable that they should constitute an essential part of the biliary drainage system, because their supposed possible cystical transformation is never followed by a secondary response from the parenchyma which always has a normal appearance (*Sherlock* (12))

It has been suggested that an overproduction of ducts is significant in the pathogenesis and that abnormal proliferation of ductal cells is the fundamental lesion of the disease (*Rumler* (10)) This conception is in part shared by *Norris and Tyson* (8) who believe

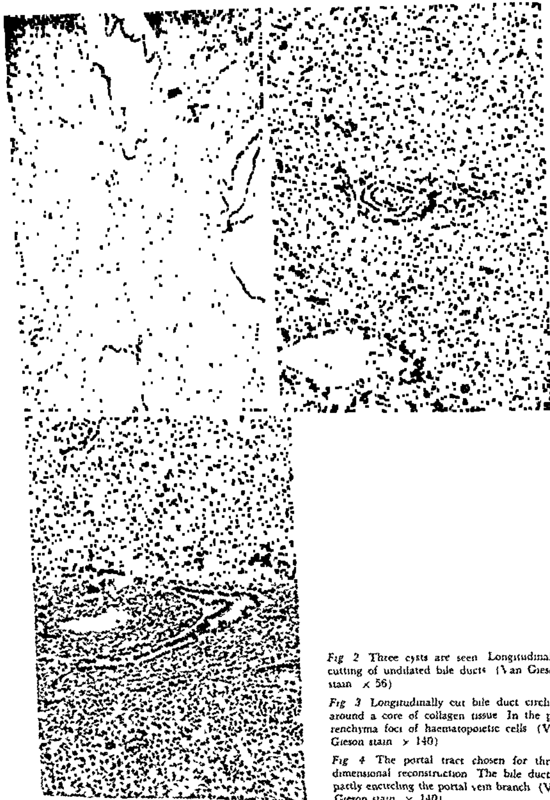


Fig 2 Three cysts are seen. Longitudinally cutting of undilated bile ducts (Van Gieson stain × 56)

Fig 3 Longitudinally cut bile duct circling around a core of collagen tissue. In the parenchyma foci of haematopoietic cells (Van Gieson stain × 140)

Fig 4 The portal tract chosen for three-dimensional reconstruction. The bile duct is partly encircling the portal vein branch (Van Gieson stain, × 140)



Fig 5 The reconstructed block The upper half of the picture is the top of the block which corresponds to the section in Fig 4 The central grey structure is the portal vein branch

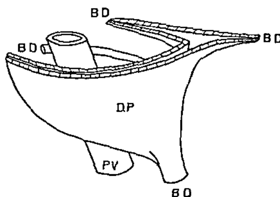


Fig 6 Simplified drawing *made from the* reconstructed block to illustrate the ductal plate All the small bile ducts originating from the ductal plate are omitted DP – ductal plate PV = portal vein branch BD = bile duct

that preceding abnormalities of the developing bile ducts might stimulate the production of excessive numbers of elements" As an overproduction of duct elements in this case can not be referred to postnatal life, it must take place during the development of the intrahepatic bile duct system or later in intrauterine life The question is therefore intimately connected to liver embryology Unfortunately our knowledge of this is insufficient Although a matter of dispute, the best synthesis of our present knowledge is probably that of *Elias* (1) According to him the duct system inside the liver in a very early phase has the form of a plate It is the *limiting plate* of the muralium duplex or multiplex bordering the invading mesenchyme along the portal vein branch In this plate, tubules are formed Some of these mature, separate from the plate and become the later interlobular ducts. This process may go wrong and the whole plate or parts of it may be separated from the rest of the parenchyme by mesenchyme which is believed to be the factor or connected to the factor inducing the transformation of parenchymal cell to ductal cell

If we turn to the case presented here, the essential finding is ' the ductal plate form of the portal bile duct submitted to reconstruction The two cases, proved by reconstruction by the author to have this architectural form, have both been newborn children and the condition therefore necessarily congenital The possibility of reactive bile duct proliferation in later life taking this ' foetal form can of course not be excluded, but seems unlikely The histological feature which is characteristic of the plateformed bile system is the lon gitudinally" cut bile ducts These have never by the author been observed in tissue taken from patients other than children and very young individuals nor are they mentioned in the literature Occasionally a bile duct can be found cut longitudinally but a picture in which all bile ducts are cut longitudinally is statistically incompatible with a tubular structure of the bile system, and must therefore be interpreted as above Until the opposite is proved it seems justified to consider the ductal plate system as evidence of congenital disorder

The plate form is found in infants and young adults An explanation of this could be that these patients die young, probably of concomitant malformations affecting systems other than the biliary and incompatible with prolonged life Another possibility is that the plates break down and disappear in the course of time Some support for this assumption may be found in the facts that the plates always show degenerative changes and continue in tubular ducts some of which are discontinuous or blind ending The plate form is seldom – if ever – met in cases of polycystic disease of the liver in older persons

Via this process of degeneration segmentation and resorption starting in intrauterine life and continuing after birth the necessary material for the cystic changes of the liver could be provided

The anatomy of the developed cysts has been described by *Norris and Tyson* (8) Their case 1 was a newborn boy with polycystic disease of the liver and kidneys Liver histology was identical with the findings in the present case A graphical reconstruction of some of the cystic elements was made and confirmed the tendency to encircle the vessels The cystic elements had numerous tubular distorted but undilated branches some of which ended blindly In the upper part of their reconstructed model the plate form of the cystic element is evident The anatomy of the cysts is much more logical and easily understood if they are developed from laminae or plates and not from tubular structures

Von Meyenburg complexes (6) are occasionally found in otherwise completely normal livers predominantly in older persons and at subcapsular sites They are often noticed in polycystic disease of the liver (*Melnick* (5)) and the cysts are believed to originate from them According to *Scheuer* (11) occasional multiple lesions are associated with portal hypertension and these cases merge imperceptibly with congenital hepatic fibrosis

In conclusion the following hypothesis is proposed for further investigations

If the primary malformation – the ductal plate is generalized throughout the liver, the result will be a generalized hepatic disorder

In one form the connective tissue in the portal tracts is very abundant so that fibrosis of the liver is a prominent pathological feature In a way not clearly understood this form of fibrosis is often accompanied by portal hypertension perhaps occasioned by fibrous compression of the vessels (*Melnick* (5)) perhaps by a deficiency in the terminal branches of the portal vein (*Kerr et al* (4)) The degradation of the ductal plate has only a minimal tendency to cystic changes. In this form the diagnosis must be called *Congenital hepatic fibrosis*

In another form the amount of connective tissue is sparse and the intrahepatic vessels are normal Portal hypertension is very rare (*Melnick* (5)) In this form the degradation of the ductal plate has a pronounced tendency to cystic changes. The diagnoses should be called *Polycystic disease of the liver*

If the primary malformation is focal the result will be scattered von Meyenburg complexes or solitary cysts without clinical interest

The ductal plate malformation is often found together with malformations of other organ systems especially the kidneys But the disorder does not affect the normal embryological development of the rest of the liver, nor does it interfere with the normal function of the biliary system

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The author wishes to express his thanks to Dr *Inge Tygstrup* head of the paediatric section in the University Institute of Pathological Anatomy Copenhagen for permission to study the case

This work was supported by a grant from *Kobmand i Odense Johann og Hanne Weimann f Seedorffs legat*

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Acta path microbiol scand Section A 80, 207-210, 1972

DISAPPEARANCE OF THE ANTI-ANAEMIA LIVER PRINCIPLE FOLLOWING DISTAL RESECTION OF THE SMALL INTESTINE IN PIGS

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After operative elimination of the distal two thirds of the small bowel young pigs developed, in the course of some months, a condition similar to that previously induced by total gastrectomy In these pigs too, a disappearance of the anti anaemia principle was demonstrated in extracts from the liver Only this particular change will be discussed in the present paper It is presumed to be due to the removal of a selective absorption area

In the efforts to elucidate the causal factors of "the experimental gastroprival symptom complex" (*Petri*), the elective resections of the stomach and duodenum (7, 13) were extended to comprise also the small bowel Isolated resection of the distal two-thirds of the small bowel in puppies – to some extent unlike elimination of its proximal segment – has resulted in the development of a complex syndrome This symptom complex was similar to that previously induced by total gastrectomy, but was besides in a way suggestive of sprue (10, 11) These changes in dogs have been subjected to more comprehensive and detailed studies (5)

Resection of the distal part of the small intestine has now been performed also on pigs which we have preferred as experimental animals for many years, int al for purposes of comparison The present report is concerned only with the anti anaemia liver principle which has not previously been studied in this way in pigs

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PREVIOUS INVESTIGATIONS

Isolated small bowel resection on pigs has previously been performed to a varying extent on a small number of animals, and only by a few investigators, in vain attempts at inducing anaemia. *Bence* (1) removed 3 m of ileum and jejunum of a 3 month-old pig Polycythaemia developed in the course of 3 months The observation period was 9 months *Brown* (2) removed almost all, or part of, the small intestine in 3 pigs The resections did not lead to diarrhoea, anaemia, or paralysis The observation periods are not stated

Geiger et al (3) removed the proximal 15 feet of intestine from a hog to determine whether the duodenum is a source of the intrinsic factor Within 8 months the liver's content of anti anaemia principle had diminished but in less degree than after total gastrectomy

PRESENT INVESTIGATIONS

Experimental animals Two pigs, Danish land race, ♀♀, Nos 174 and 177, 11 weeks when operated upon, then weighing 20 and 20 kg and 80 and 78 cm in length respectively Another 3 pigs survived the operation for only 9 days, 2 months, and 2½ months respectively, and therefore they were left out of the analysis

Anaesthesia Ether after injection of sodium phenobarbitone

Operation In both pigs resection of the distal 8 64 m (64 per cent) of the small bowel which was a total of 13 44 m measured *in situ*, from the duodeno-jejunal flexure to the distal 5 cm of the ileum where the anastomosis was established, side to side

Experimental conditions, incl diet as described previously (6, 7, 12, 14)

Observation period 8½ months The pigs were killed by exsanguination under anaesthesia

Clinical and morphological findings were similar to those previously induced by total gastrectomy These findings will be discussed later and compared with those in dogs After resection of the small intestine, however, the stunted pigs did not develop anaemia of a macrocytic, hyperchromic nature, but a certain tendency to polycythaemia The diarrhoea in the pigs was merely initial, transient, and short-lasting Terminal weight and length 28 and 28 kg, 81 and 84 cm respectively The proximal segment of the small bowel which was left behind measured 4 8 m, as found originally *in vivo* – neither lengthened nor dilated The duodenum and stomach were grossly normal

Extract of the liver (Hepsol 6–87 and Hepsol 6–88) from the two experimental pigs (Nos 177 and 174) was prepared in the same way as previously (8, 9) Either one or the other of the extracts was injected into 3 patients with pernicious anaemia 10 ml i m daily for two days In a number of other patients the severity of the disease did not permit continuation of the trial After an interval of 12 days, a corresponding dose of the control preparation, ordinary Hepsol (MCO) was injected i m All three patients were elderly, having typical, previously untreated pernicious anaemia As an *indicator* of the aetiological influence of the operative procedures upon the quantity of anti-anaemia principle in the liver, the effect of liver extract upon patients with pernicious anaemia is still used This attitude will continue as long as our investigations into the absorption and deposition of B_{12} in the operated pigs have *not* been concluded and as long as it has not been

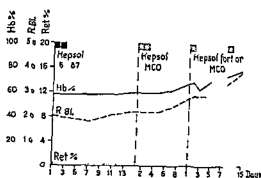


Fig 1 (Case 1) Absence of haematological reaction in a patient with pernicious anaemia following i m injection of liver extract (Hepsol 6–87) from an ileum resected pig On the other hand, a positive haematological responce to subsequent injection of a control preparation (Hepsol MCO)

fully elucidated whether or not the effect of the liver extract is conditioned exclusively by B_{12} (15)

Case 1

I F, male aged 67, admitted to Medical Dept III, Kommunehospitalet, Copenhagen, on 14 11 1946 and discharged on 23 1 1947

Untreated pernicious anaemia of three months' duration Recurrent attacks of renal calculi through 35 years

Fatigue, weight loss, paraesthesiae in the smooth tongue, hands, and legs, mild depressions Hb 57 per cent, R B C 2 08 mill, colour index 1 4, W.B C 4800 (lymphocytes 35 per cent) Considerable poikilo and megalo cytosis Histamine test Congo O W R negative

Treatment 1 Hepsol 6–87, 10 ml i m, daily for two days (23 11 and 24 11) 2 After an interval of 12 days Hepsol MCO, 10 ml i m daily for two days (7 12 and 8 12) 3 After another interval of 7 days Hepsol high potency MCO 5 ml i m weekly

Case 2

I E M P, female aged 57 admitted to Medical Dept III, Kommunehospitalet, Copenhagen, on 15 3 1947 and discharged 17 4 1947

Untreated pernicious anaemia of four months' duration

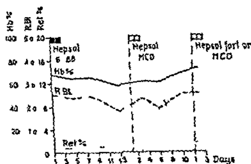


Fig 2 (Case 2) Same findings as in Fig 1

Slight jaundice, fatigue, weight loss, paraesthesiae of the tongue Hb 67 per cent, RBC 2 5 mill, colour index 1 3 WBC 3600 (lymphocytes 37 per cent), reticulocytes 0 8 per cent, diameter of red cells 5 4–11 5μ Considerable aniso and poikilocytosis, numerous microcytes and megalocytes Histamine test Congo 0 WR negative

Treatment 1 Hepsol 6–88, 10 ml i m, daily for two days (20 3 and 21 3) 2 After an interval of 12 days Hepsol MCO 10 ml i m for two days (3 4 and 4 4) 3 After another interval of 9 days Hepsol fortior MCO 10 ml i m for two days

Case 3

G M C female aged 45, admitted to Medical Dept III Kommunehospitalet Copenhagen on 18 4 1947 and discharged on 21 6 1947

Untreated pernicious anaemia of one year's duration History of operation for Graves' disease 15 years previously Still nervous

Fatigue weight loss, paraesthesiae of smooth tongue paraesthesiae and spasms of the limbs Hb 49 per cent, RBC 1 9 mill colour index 1 2, WBC 4800 (lymphocytes 46 per cent), reticulocytes 0 6 per cent Considerable aniso-, poikilo- and megalocytosis Histamine test Congo 0, WR negative

Treatment 1. Hepsol 6–87, 10 ml i m daily for two days (26 4 and 27 4) 2 After an interval of 12 days Hepsol MCO, 10 ml i m daily for two days (10.5 and 11 5). 3 After another interval of 11 days Hepsol high potency MCO 5 ml i m weekly, the first dose on 23 5 Also iron medication

RECAPITULATION AND DISCUSSION

Two young pigs, both aged 11 weeks, were subjected to resection of the distal 8 6 m (64 per cent) of the 13 4 m long small bowel The pigs were observed for 8½ months before being killed Liver extract from the experimental pigs (Hepsol 6–87 and 6–88) injected i m into three patients with pernicious anaemia did not result in any specific haematological reaction Administration of a control preparation (Hepsol MCO), on the other hand, was followed by a haematological response in all three patients

Thus elimination of the distal segment of the small bowel in pigs has (among other things) compromised the presence of the anti-anaemia principle in the liver

If the quantity of this principle had merely diminished, the operation would have to some extent reduced the conditions offered to the active principle in the gastrointestinal tract In that event, the following causes might be considered (a) Diminution of the

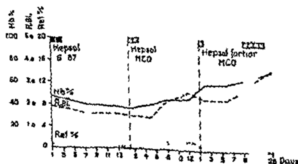


Fig 3 (Case 3) Same findings as in Figs 1 and 2

area of intrinsic factor production which certain authors believe is situated also in the small bowel (15), (b) a partial loss of a presumed retrograde stimulating impulse from the intestine to the stomach, corresponding to the tendency to "enterogenous achylia" in dogs after small intestinal resection (5, 10), or (c) the presence of some absorptive function within the proximal segment of the small bowel not directly affected by the resection

However, the disappearance of the specific liver principle in the operated pigs was total Apparently, this must be ascribed to the removal of an absorption area which is specific and strictly localized to the distal segment of the small bowel in the adult pig

In this species similar findings have been made with B_{12} In five baby-pigs orally administered radioactive B_{12} has been demonstrated in highest quantity in the mid part of the small intestine and the area distal to this part (4)

The present studies were aided by a grant from the *P Carl Petersen Foundation*

Technical aid by Mrs *Anna Jorgensen*, Miss *Ellen Margrethe Andersen* and Mrs *Elisabeth With petersen*

Translated from the Danish by Mrs *Anna la Cour*

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Acta path microbiol scand Section A 80, 211–221, 1972

THYMIC CYSTS IN OESTROGENIZED BALB/c MICE

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BALB/c male mice injected monthly with 1 mg oestradiol benzoas developed testicular tumours, a generalized lymphocyte depletion, clusters of PAS positive cells in spleen and lymph nodes, spleen amyloid and PAS positive thymus cysts Treatment of castrated males and intact females induced the same thymus cysts Thymectomy at three days of age aggravated the lymphopenia, reduced the survival time and accelerated spleen amyloid development in oestrogen treated male and female mice Testicular tumours appeared in some thymectomized males All thymus cysts contained gamma globulin and albumin A lymphocytestimulating factor was not found in thymus extracts of treated mice and Coombs test and indirect fluorescence tests for autoantibodies were negative Electron microscopic findings were consistent with ongoing transport by pinocytosis through the cells lining the thymus cysts

Long-term treatment of male BALB/c mice with a oestrogen induces testicular tumours and general lymphocyte depletion (8)

It was previously shown that BALB/c mice after 12 months of oestrogen treatment also may have large thymic cysts (11)

In the present work the effect of castration and thymectomy on the development of thymic and extrathymic lesions was examined Thymus lesions were also examined by fluorescence microscopy and electron microscopy

MATERIAL AND METHODS

Inbred BALB/c mice were used Thymectomy was performed at 3 days of age and castration at 2 months Oestradiol benzoas in aqueous suspension (Ovex Lovens Kemiske Fabrikker, Ballerup, Denmark) was administered once a month from 2 months of age by injecting 1 mg subcutaneously (sc)

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14*

Oestrogen treated mice were killed when moribound Control animals were given saline sc and killed shortly after the oestrogen treated mice Lung, liver, spleen, kidney, peripheral lymph nodes, thymus and thyroid gland were taken for microscopy and stained with haematoxylin-eosin and periodic acid-Schiff (PAS) and in selected cases with alcaline Congo red, Sudan black and toluidine blue Spleen amyloid was graded 1 6 Three indicating an unbroken ring of amyloid around the folicles, six a conversion of the whole organ into amyloid (5) Blood was taken for leucocyte counting and haematocrit evaluation and for electrophoresis on cellulose acetate (24) A few mice received 10 mg carbon particles intravenously (2) 3 days before they were killed

Both direct and indirect *immunofluorescent techniques* (29) were employed Fluorescein thiocyanate (FITC) labelled rabbit anti mouse IgG antiserum (Hyland Div Travenol Lab Inc, Los Angeles Lot No 82226 DOO2A1) diluted 1 4 in Coon's buffer served as antiserum In immuno electrophoresis undiluted antiserum gave a gamma precipitation and a trace reaction in the α_2 region with whole mouse serum and mouse gamma globulin type G (26) When diluted 1 4 with Coon's buffer, a monospecific reaction with whole

TABLE 1 *Influence of Treatment with Oestrogen on Intact, Castrated and Thymectomized BALB/c Mice*

Sex	Treatment	Number of mice	Survival time in months		Spleen amyloid**		Leuka
			Mean	Range	Mean	Range	Thymus
♂	Oestrogen sc	26	9 1	(1–15)	1	(0–3)	5
	NaCl sc *	14			0 3	(0–2)	0
	Oest sc + Castration	9	8 3	(1–13)	1	(0 3)	2
	NaCl + Castration	8			0		
	Oestrogen + Thymectomy	10	6 5	(4–12)	1 5	(0–3)	
	NaCl + Thymectomy	10			0		0
♀	*Oestrogen*	*10*	*6 3*	(4–10)	2	(0–3)	0
	NaCl	10			0		0
	Oestrogen + Thymectomy	8	4 5	(4–6)	3 3	(2–3)	0
	NaCl + Thymectomy	9			0		0

* Saline treated mice were killed when the matching oestrogen treated animals had been s rificed

** Graded 1–6 Six indicating a conversion of the whole organ into amyloid (5)

mouse serum occurred In the indirect technique the following antisera from Central Laboratorium van de Bloedtransfusiedienst van het Nederlandsche Roede Kruis were used A FITC labelled horse anti rabbit immunoglobulin (PK 17–2F), a rabbit anti mouse IgG antiserum (KM 16-6-PI) con *taining traces of antitransferrin and antialbumin*, and rabbit anti mouse albumin (KM 2–1–PA) which gave a monospecific reaction with whole *mouse serum in immunoelectrophoresis*

Serum from human subjects and from NZB mice with and without anti nuclear factor (ANF) served as controls for the presence of ANF*

Newborn BALB/c and C3H mice were used in *the assay for lymphocyte stimulating factor* (LSF) (20)

The *cytotoxic test* was made according to *Gorer & O Gorman* (15), the *direct Coomb's test* according to a previously described technique (10) Leucocytes and *red blood cells* incubated with isoimmune sera from C57BL mice immunized with BALB/c spleen cells served as positive controls for the Coomb s and the cytotoxic tests

* Advice on fluorescence technique was kindly given by Dr *P Elling* the State Serum Institute, Copenhagen

Electron Microscopic Technique

Blocks of thymus tissue were fixed for 2 hou in 3 per cent glutaraldehyde (0 15 M phospha buffer pH 7 1) and for one hour in 2 per ce osmium tetroxide (0 15 M phosphate buffer p 7 1) They were then dehydrated in acetone an embedded in Vestopal W Areas for ultramicrotom were chosen by light microscope examination c 1 micron sections which had been stained wit toluidine blue

Ultrathin sections were cut on an LKB Ultro tome stained with magnesium uranyl acetate (14 and lead citrate (25) and examined in a Siemen Elmiscope I

RESULTS

After a few months of treatment with oestrogen the mice wasted and showed allopecia on the backs Some died with diarrhoea, especially the females Repeated *leucocyte counts* on intact oestrogen treated males demonstrated a drop in leucocyte value from the normal 4–5000 to about 2500 cells/mm³ after the third month of treatment 42 intact and 9 castrated males treated with oestro-



Fig 1 PAS stained section of thymus from BALB/c male treated with oestrogen for 10 months A marked depletion of thymus lymphocytes is combined with the occurrence of numerous cyst-like structures staining positively with PAS PAS technique × 210

gen exhibited leucocyte values at time of death of 2459±249 ($p < 0.01$) and 2302±194 ($p < 0.01$), respectively, in contrast to 1506±38 ($p < 0.01$) for 10 thymectomized mice and 4200±199 cells per mm³ for 24 healthy intact male and female controls (group of reference) Both the tibia and femur marrow cavities were much reduced in all groups after a few months of treatment due to a thickening of the bone Most non-castrated oestrogen treated BALB/c males whether unoperated or thymectomized, developed interstitial cell *testicular tumours*

The mean *survival time* of intact males was slightly longer than that of castrated males and definitely ($p < 0.01$) longer than that of intact females Thymectomized animals revealed additional reduction in survival time (Table 1) When compared to untreated mice (11) all groups of oestrogen treated mice showed a significant ($p < 0.01$) reduction in survival time (Table 1)

Spleen amyloid was more frequently found in oestrogen treated non-operated males and females and castrated males than in saline treated controls Thymectomy accelerated the amyloid development in both sexes (Table 1) The amyloid stained with alcaline Congo red

Haematocrit values and *electrophoresis* on cellulose acetate were found normal in all groups of BALB/c mice None of the 51 saline treated mice, but 7 out of 45 oestrogen treated non thymectomized mice developed *thymic lymphoma* with heavy infiltration of the enlarged thymuses and peripheral leucocyte counts of between 10^4 and 10^5 cells per mm³ (13) Cysts were seen in some leukaemic thymuses

Irrespective of the presence or absence of testes the *light microscopic thymus lesions* were the same in all oestrogen treated mice After six months of treatment the number of

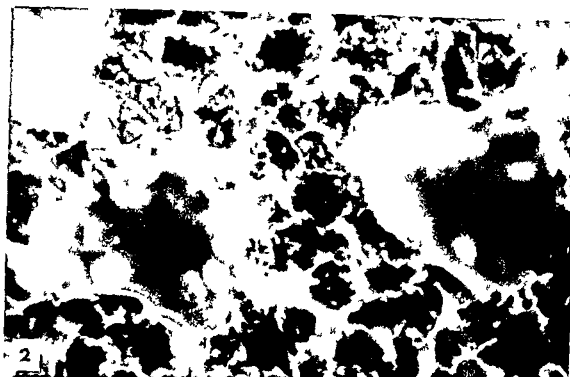


Fig 2 Greater magnification of the thymus from the same mouse as depicted in Fig 1 Note numer ous cell size globules in the cysts PAS technique × 2100

cortical lymphocytes was greatly reduced and the remaining lymphocytes sometimes showed signs of necrosis Cortical PAS cells increased in number with continued oestrogen treat ment except for a final decline in old mice PAS stained sections revealed intensely stain ing medullary cysts especially in 10–12 month old mice (Fig 1) All cysts stained slightly positive with toluidine blue and ne gative with Sudan black Some cysts appar ently contained a structureless material, whe reas in other cases a sub structure of cell sized globules was detectable (Fig 2) Some PAS cysts contained nuclei sized clumps which could be stained with Feulgen and with Unna Pappenheim Intravenously ad ministered carbon did not aggregate in the cysts Control mice 12 months of age exhibited some cortical PAS cells and a moderate degree of lymphocyte depletion PAS cysts in the me dulla were extremely rare in saline treated controls, though thymuses of 20–24 month old untreated mice did exhibit a few cysts

Using the indirect *immunofluorescent* tech nique on sections from 12 oestrogen and 6 sa line treated 8 to 12 month old male mice a bright fluorescence was observed in all the PAS positive thymus cysts with anti gamma globulin and with anti albumin Sometimes the fluorescence was totally homogeneous so metimes it appeared as several fluorescent globules superimposed on each other (Fig 3) When examined in the phase contrast mi croscope the fluorescent areas seemed to ex hibit a sub structure (Fig 4) Fluorescence in the thymus outside the cysts was never ob served Clusters of PAS positive cells (Fig 5) which occured in the spleen and periphe ral lymph nodes of oestrogen treated mice exhibited membrane fluorescence (Fig 6) while saline treated controls exhibited only a few isolated fluorescent cells in these or gans Liver diaphragma and testes were free of fluorescence Moderate fluorescence was seen in kidney glomeruli

Incubation of sections with non labelled antiserum prior to incubation with FITC labelled anti gamma globulin eliminated

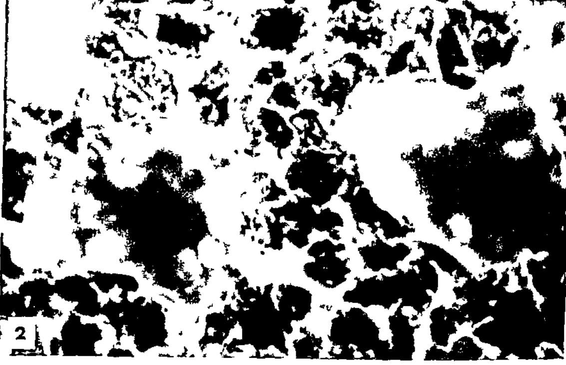


Fig 2 Greater magnification of the thymus from the same mouse as depicted in Fig 1 Note numerous cell size globules in the cysts PAS technique × 2100

cortical lymphocytes was greatly reduced and the remaining lymphocytes sometimes showed signs of necrosis Cortical PAS cells increased in number with continued oestrogen treatment except for a final decline in old mice PAS stained sections revealed intensely staining medullary cysts especially in 10–12 month old mice (Fig 1) All cysts stained slightly positive with toluidine blue and negative with Sudan black Some cysts apparently contained a structureless material whereas in other cases a sub structure of cell *sized globules was detectable (Fig 2) Some* PAS cysts contained nuclei sized clumps which could be stained with Feulgen and with Unna Pappenheim Intravenously administered carbon did not aggregate in the cysts Control mice 12 months of age exhibited some cortical PAS cells and a moderate degree of lymphocyte depletion PAS cysts in the medulla were extremely rare in saline treated controls though thymuses of 20–24 month old untreated mice did exhibit a few cysts

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Incubation of sections with non *labelled* antiserum prior to incubation *with FITC-*labelled anti gamma globulin *eliminated*



Fig 7 Part of an epithelial thymus cy · uogen for 10 months The epithelial cells (EP) are joined l ,mphocyte (LY) is found between two epithelial cells Except for cell deb ,at content (CC) is a homogeneous and moderately electron dense mass × 8000

endoplasmic reticulum than cells without granules

DISCUSSION

Intact males seem to be more resistant to wasting and early death than castrated males and intact females and this would indicate some pathogenic importance of testicular hormone (19) The immediate , of death might be infect as cas hoea were seen esp mon living females Most ic lymphoid cells is int p ing to death as thym hi in additional lyt o further reduction

Thymectomy

duced amyloidosis (3) and this is in accordance with reports on casein induced amyloid in adult (23) and newborn (12) thymectomized mice In both papers referred to the accelerated amyloid development was tentatively linked to the immune depression which follows thymectomy

The PAS cells clustering in the spleen and lymph nodes may be clones of rapidly dividing cells vulnerable to the known growth inhibiting effect of oestrogen (18)

The occurrence of nuclear material, gamma globulin and albumin in thymus cysts also be a result of the growth inhibit- ct of oestrogen leading to cell death er a passive infiltration of serum (16) Thymus cysts might also be a

Fig 5 Spleen section from male mouse treated with oestrogen for 11 months Clusters of cells sta ing homogeneously with PAS are present in the perifollicular zone A depletion of normal lymph cytes is apparent PAS technique × 240

Fig 6 Cryostat section of the spleen shown in Fig 5 Incubation with FITC labelled anti mou gamma globulin causes fluorescence of several clusters of cells A similar fluorescence was obta n with anti albumin The fluorescent cells seemed to be identical with the intensely PAS positive ce demonstrated in Fig 5 PAS technique × 2100

thymus extract from 5 oestrogen treated and 6 control mice was found

The epithelial thymus cysts in male mice treated with oestrogen for 10 months contained a *moderately electron dense material* of fine granular or fibrillar texture (CC) (Figs 7, 8 9) In addition some of the cysts displayed cell debris (DB) (Fig 7)

The epithelial lining cells were firmly attached to each other as plasma membranes of adjacent cells displayed lateral foldings (LF) (Fig 8) and desmosomes (DM) (Figs 7, 8) Tight junctions were never observed The lining cells thus completely separated the cyst content from the surrounding connective tissue space (TS) (Fig 9) In some cases, however just a thin layer of cytoplasm separated the two (EP) (Fig 9)

Small lymphocytes were occasionally noted between epithelial lining cells (LY) (Fig 7) but they were never in contact with the cyst lumen

Some epithelial cells displayed microvilli at the luminal surface (VI) (Fig 8), and sma pinocytose vesicles (PV) (Fig 9) were ofte noted in conjunction with the plasma mem brane

The epithelial cells contained *few* cyto plasmic organelles Mitochondria were few and cisterna of the granular endoplasmi reticulum were noted only occasionally Mos of the cytoplasmic ribosomes were polyribo somes

A large Golgi zone was sometimes observed usually surrounded by coated (CV) (Fig 8) and uncoated vesicles (MV) (Fig 8)

A low percentage of the lining epithelial cells contained electron dense cytoplasmic granules The granules were limited by a tripple layered membrane and varied in size *from half a micron to several microns*

The larger granules occasionally contained *myelin like material*

Epithelial cells with electron dense granules in the cytoplasm displayed more granular



Fig . . . the same mouse as shown in Fig 8 is separated fror . . . . . thin layer of epithelial cell cytoplasm (EP) The base men . . . ly distinct Pinocytose vesicles (PV) are formed from the "outer" and . membrane Coated vesicles are marked CV × 40,000

result of a more specific stimulating effect of the oestrogen on a function of the thymus medullary cells as these often contain a PAS positive substance (6) and good evidence of thymus ovarian interaction exists (21) Thymus cysts are hardly a break down product of particulate material phagocytized by the lining cells, as no accumulation of intravenously injected carbon was demonstrable The emergence of PAS cysts *in very old*, untreated BALB/c mice (27) might be related to hormonal disturbances following the adrenal hyperplasia which occurs in many old mice of this strain (9) Cysts are also found in old mice of other strains (1)

The lack of a lymphocyte stimulating effect of thymus suspensions from oestrogen treated mice means that the cysts at the stage investigated do not represent a reservoir of the lymphocyte stimulating hormone (20), and the possibility that the thymus lesions were reflecting an auto immune disorder (4, 17, 28) was not born out in our experiments

From the electron microscopic examination it is apparent that the cyst content is completely separated from the wide-spread connective tissue space by lining epithelial cells and basement membranes

We found no morphological evidence of secretory activity confined to the epithelial cells The numerous small pinocytic vesicles i the epithelial cell cytoplasm, however, sug-gest a vivid trans-epithelial transport either from the connective tissue to the cyst lumen or *vice versa*

The finding of cell debris in the epithelial cell cysts explains the shadows observed by light microscopy The micrographs, however, do not reveal the origin of the cell debris

Van Giesson staining has demonstrated abundant connective tissue in thymuses of oestrogen treated mice (11) The electron microscopic findings of the present investigation are consistent with the concept of a widespread connective tissue space

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This investigation was supported by grants from *Daell Fonden, Anders Hasselbalcks Fond til Leukaemiens Bekaempelse*, and the *Danish Medical Research Council* The Laboratory of Tumour Virus Research is supported by grants from the *Danish Cancer Society*

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EXTRACARDIAC RHABDOMYOMA

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A case of a rhabdomyoma of the neck tissues is described The tumour was accidentally found during an autopsy in a 78 year old woman The tumour showed histologically classical patterns without any evidence of malignancy The problems of differential diagnosis are discussed

Extracardiac rhabdomyoma is very rare *Moran and Enterline* (1964) gathered 11 acceptable cases from the literature to which they added one of their own After the first electron microscopic study by *Cornog and Gonatas* (1967) further cases with ultramicroscopic studies have been described by *Battifora & al* (1969), *Kay & al* (1969) and *Wyatt & al* (1970) By 1970 18 cases could be traced in the literature

Judging from the scanty reports available, the tumour appears to be equally common in both sexes In 15 of the above 18 cases the tumour was situated in the throat or oral cavity and thus appears to show a definite predilection for these areas The patients' ages ranged from 8 weeks to 81 years (*Czernobilsky & al 1968*) *Goldman* (1966) described a multicentric rhabdomyoma The tumours have been benign and have never been fatal No metastases have been reported The fact that the tumour arises most often in the pharynx and throat together with its benign behaviour probably explain why the lesion is not included in autopsy statistics

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CASE REPORT

A woman, aged 78, died with clinical symptoms of senile dementia and urinary tract infection Autopsy revealed generalized arteriosclerosis, encephalomalacia, nephrolithiasis with chronic pyelonephritis

No metastases of this tumour were found Adjacent to the left thyroid lobe was an encapsulated, fusiform tumour The tumour which had not involved the large cervical blood vessels was readily enucleated The thyroid was small and contained some colloid adenomas

Pathology The tumour measured $7 \times 2 \times 2$ *cm*

nation revealed very large, polyhedric tumour cells with oxyphilic fine granular cytoplasm (Fig 2) The cytoplasm stained violet with *Masson's* trichrome stain and was PAS negative The vesicular nuclei were situated peripherally in the cells and had small nucleoli No nuclear inclusions were seen Many of the tumour cells contained 2 or 3 nuclei Some tumour cells were elongated and contained multiple nuclei which tended to be arranged in rows Other cells contained clusters of nuclei resembling what may be seen in damaged striated muscle All the cells had well defined membranes Many cells had cytoplasm with large vacuoles whose content was PAS negative The oxyphilic cytoplasmic masses, however, contained scattered clusters of small, strongly PAS positive particles In occasional tumour cells the cytoplasm exhibited transverse striation most distinctly seen with *PATH* stain (Fig 3) The stroma cells were scarce and except around blood vessels no reticulin was seen The



Fig 1 Macroscopic appearance of the tumour (MS 335/70) Scale 1 1



Fig 2 Large, often vacuolated tumour cells with single or multiple nuclei (Hematoxylin and eosin × 320)

tumour contained however, abundant capillary vessels In the periphery of the tumour were a few isolated fibres of normal striated muscle tissue The capsule consisted of a thin collagen membrane Single well defined lobules of tumour tissue were seen on the outer surface of the capsule Electron microscopy on the tumour has been performed The cytoplasmatic and nuclear structures were, however, too insufficiently preserved to be discussed



Fig 3 Cross striation in cytoplasm (PTAH, × 800)

DISCUSSION

Rhabdomyoma shows a striking predilection for the throat, pharynx and oral cavity No explanation for this can be offered The number of cases on record is, however, small, and it is possible that this tendency will be less marked in larger series This localization, however, raises important differential diagnostic problems especially in the examination of frozen sections of biopsy specimens The benign appearance of the tumour distinguishes it clearly from malignant oxyphilic tumours such as rhabdomyosarcoma and large celled squamous cell carcinoma Renal cancer, which sometimes metastasies to the thyroid, may be more difficult to exclude The tumor should be differentiated from chemodectoma and oxyphilic adenoma originating in the thy-

roid or parathyroid It should be stressed that rhabdomyoma shows no alveolar arrangement of the reticular fibres and that it is very poor in reticulin Rhabdomyoma differs clearly from so called granular cell tumour ('myoblastoma'), especially in its electron microscopic picture (*Battifora* 1969) In some respects rhabdomyoma of striated muscsle and of the myocardium are very similar but it is not certain whether it is a question of the same tumour This problem has been thoroughly discussed by *Moran & Enterline* (1964) and *Czernobilsky et al* (1968) The striation of cell cytoplasm is more striking in cardiac rhabdomyoma, but needle-like, PAS positive formations of irregularly arranged Z band like structures have been described only in extracardiac rhabdomyoma Cardiac rhabdomyoma occurs in about 50 per cent of all cases with tuberous sclerosis and other congenital malformations None of the cases of extracardiac rhabdomyoma reported have shown such an association

Thanks to the characteristic morphology of rhabdomyoma the tumour seems to be easy to diagnose histologically provided this rare type of lesion is borne in mind by the pathologist

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Acta path microbiol scand. Section A 80, 225-234, 1972

ENDOCRINE CELLS IN THE BRONCHIAL MUCOSA OF HUMAN FOETUSES

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Cells which are believed to be members of the APUD-series of endocrine cells have been demonstrated in the bronchial epithelium of foetal lung Among the common cytochemical characteristics of APUD-cells were demonstrated amine content, amine precursor uptake (and decarboxylation), and reactivity to staining with HCl toluidine blue and Pb-H

Feyrter (1953) introduced the term "paracrine glands" to characterize a system of scattered endocrine cells observed in exocrine and endocrine organs The paracrine cells were believed to have an exocrine as well as an endocrine function

Pearse (1969 a) used the term APUD cells to characterize a similar system of cells sharing a number of cytochemical and ultrastructural characteristics They were given the name APUD cells because their most constant cytochemical characteristics were *A*mine content, amine *P*recursor *U*ptake and *D*ecarboxylation A common function of the APUD cells is probably secretion of low molecular weight polypeptide hormones The hormones are believed to be stored in *secretory granules, but* its synthesis in the cells has not yet been proven in details

The Paracrine cells of human lung were described as clear , empty appearing cells located basally in the epithelium lining the respiratory tract (*Feyrter* 1954) In foetal life as well as in childhood some of these cells were argyrophilic but in adults argyrophilia could not be demonstrated

The present study is concerned with the cytochemical charactetristics of non-ciliated cells in the bronchial epithelium of the human foetus Especially on the basis of their amine content and their uptake of amine precursor, these cells are thought to be members of the APUD cell group

MATERIAL AND METHODS

The material comprised 31 human foetuses removed by Caesarean section in connection with legal abortion Crown rump length of the examined foetuses varied from 40 mm to 190 mm (Table 1), corresponding to a menstrual age ranging from the loth to the 23th week

Measurements were made on unfixed foetuses held in a supine position

Fixation Foetuses were fixed by whole body perfusion through the umbilical vein in order to make it possible to perform electronmicroscopic examination too As perfusate was used ice cold 3 per cent glutaraldehyde in 0,2 M cacodylate buffer at pH 7 4 After perfusion samples of lung tissues were fixed further by immersion in ice cold 6 per cent glutaraldehyde in 0,2 M phosphate buffer at pH 7 4 or glutaraldehyde picric acid, or 10 per cent neutral buffed formaldehyde or Bouin's fluid However, in some cases pulmonary tissues were fixed only by immersion or after freezedrying

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TABLE 1 *Methods Applied to Foetuses of Varying Chown rump Lenght*

Crown rump lenght (mm)	Foetus number	Pb-H	HCl Toluidine blue	Davenport silver nitrate	Grimelius silver nitrate	Bodian (Singh) silver protein	Bodian (Grimelius) silver protein	Fluorescence + L DOPA	Fluorescence —L DOPA	Diazonium reaction	Zanthydrol reaction	Masson Hamperl's me. Lod
40–59	9	×	×	×						×	×	×
	23	×	×	×	×	×	×			×	×	
	31							×	×			
	34	×	×		×			×	×			
	36	×	×		×			×	×			
	38							×	×			
60–79	5	×	×									
	6	×	×									
	11	×	×	×	×	×	×			×	×	×
	18	×	×							×		
	20	×	×	×	×	×	×			×	×	×
	33							×	×			
	35	×	×		×			×	×	×		
	39							×	×			
80–99	3		×	×								
	8	×	×	×								
	10		×	×						×	×	
	15	×		×								
	16	×	×	×	×	×	×			×	×	×
	17	×	×	×	×	×	×			×	×	×
	22											
	26	×	×		×	×	×					
	37	×	×		×			×	×	×		
100–119	4	×	×	×						×	×	
	19	×	×	×	×	×	×			×	×	×
	24	×	×	×	×	×	×			×		
120–139	14	×	×	×	×	×	×			×	×	×
	32	×	×		×			×	×	×		
140–159	1	×	×	×	×	×	×			×	×	×
	21	×	×							×	×	
160–199	30	×	×	×	×	×	×				×	×

in formaldehyde vapour The observations made on perfusion/immersion fixed tissues were identical to those made on immersionfixed tissues only

The following methods were applied to paraffine sections 1 HCl toluidine blue method (masked metachromasia) for endocrine cells according to *Solcia et al* (1968) Routinely was used 0 2 N HCl at 60° C 10 hr and toluidine blue at pH 5 o As control were used unhydrolysed sections *In a few* cases 0 5 per cent 1 per cent and 1N HCl were tested by varying the duration of action from 3 to 16 hr and tests with toluidine blue in McIlvaine's buffer at pH 3 0 and 4 0 to 6 0 were performed Glutaraldehydepicric acid was used as *fixative*

2 Lead haematoxylin (Pb H) as a stain for endocrine cells according to *Solcia et al* (1969 b)

Some sections were hydrolysed (0 2 N HCl at 60° C, 10 hr) before staining with Pb-H Glutaraldehyde 6 per cent in phosphate buffer at pH 7,4 was used as fixative

3 For detection of argentaffin cells Alkaline diazonium techniques with Fast Garnet GBC or Fast Black K, and Zanthydrol reaction (*Solcia et al* 1969 a) Glutaraldehyde 6 per cent in phosphate buffer at pH 7,4 was used as fixative Masson Hamperl s argentaffin reaction (*Singh* 1964, *Vialli* 1966) Neutral buffered 10 per cent formaldehyde was used as fixative

4 For detection of argyrophilic cells Davenport silver nitrate stain (*Hellman et al* 1960) without any addition of concentrated ammonium hydroxide as recommended by *Vassallo et al* (1969) Bielschowsky argyrophil method as modified by *Siever et al* (1965) Bodian silver protein technique (*Singh* 1962 *Grimelius* 1964), silver protein (Roques®) was used Grimelius silver nitrate stain (1968) always using incubation for 3 hr at 60° C and double impregnation Neutral buffered 10 per cent formaldehyde and Buin s fluid were used as fixative

As control tissues for methods 1 to 4 were used fundic and pyloric mucosa of mouse

5 In selected cases small pieces of pulmonary tissue were incubated in continuously oxygenated Tyrode s solution containing 25–100 mug/ml of L-d hydroxypenylalanine (L-DOPA), according to the procedure recommended by *Hakanson et al* (1969) Control pieces were incubated in Tyrode s solution alone Specimens were then washed in cold (4° C) Tyrode s solution for 1 hr and quenched to the temperature of liquid nitrogen freeze dried and exposed for 1 hr at 80° C to gaseous formaldehyde (made from paraformaldehyde) for histochemical demonstration of monoamines (*Falck et al* 1955) As control some pieces were not exposed to gaseous formaldehyde The paraformaldehyde used in this reaction was standardized in an atmosphere of constant relative humidity between 50–70° This was performed in closed vessels containing aqueous solutions of sulphuric acid between 35–46 per cent Fluorescense microscopy was carried out with a Reichert Zetopan microscope using a mercury arc lamp (HBO 2000 W) with exiter filter BG 12/3 mm or UG 1/2 5 mm [illegible]

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RESULTS

In sections of *foetal lung*, endocrine cells (APUD cells) were only identified in the bronchial mucosa They were situated basally in the epithelium close to the basement membrane The cells were oval, pyramidal or bottle shaped, and might be located in the main bronchi as well as in the intrapulmonary bronchi Occurrence of APUD cells and their reactivity to different methods was found to be independent of the age of the foetuses

With the HCl toluidine blue method the cells gave a purple to violet metachromasia (Fig 1 and 2) The metachromasia was extinquished at pH 4 4, and a rise of pH to 6 0 led to a great loss of selectivity Without acid hydrolysis, no staining of the cells could be obtained

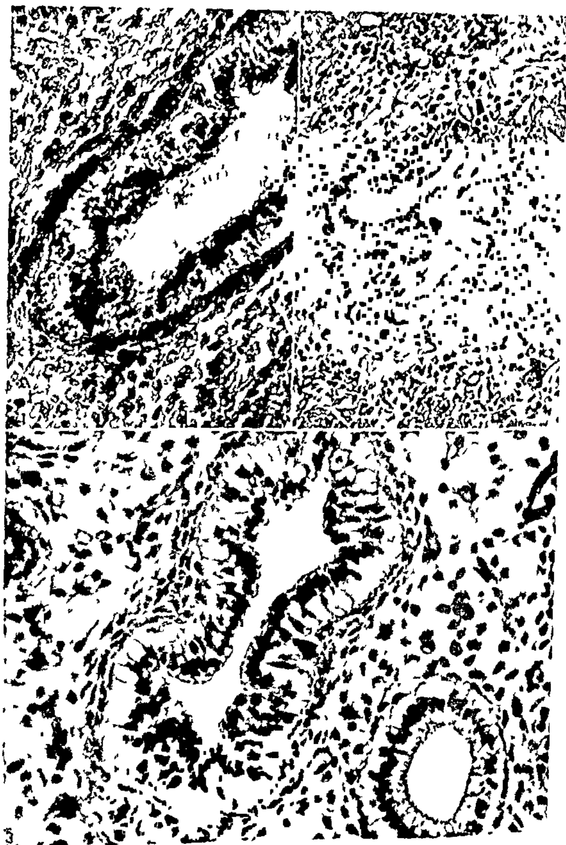
Using Pb H, few cells were stained dark blue (Fig 3) Hydrolysis before staining did not enhance the staining Cells stained by HCl toluidine blue and Pb-H were scattered, usually singly, in the bronchial epithelium

None of the chemical reactions described as positive for argentaffin cells could be observed in cells of the bronchial epithelium

Using the argyrophil silver techniques some cells were stained by Grimelius silver nitrate stain (1968) (Fig 4 and 5) If the technique of Davenport was used (*Hellman et al* 1960), a greater number of argyrophilic cells would be demonstrated (Fig 6) Argyrophilic cells occurred singly as well as in groups, mostly at the divisions of bronchi The argyrophil reaction occurred in the form of brown to black silver granules in the cytoplasm The granules were concentrated in the basal part of the cell, giving the cell an oval or pyramidal configuration

With the Bodian technique as modified by *Singh* (1962) a few scattered cells could be demonstrated, but with the Bodian technique modified by *Grimelius* (1964) and the technique of Bielschowsky (*Siever et al* 1965) argyrophilia could not be demonstrated

After incubation with L DOPA a large number of cells emitted a green formaldehyde induced flourescence, probably reflecting the presence of dopamine formed intracellulary from the administrated L-DOPA In some bronchi many cells could be demonstrated, in others none The cells were pyramidal, oval and bottle shaped often forming a shal-


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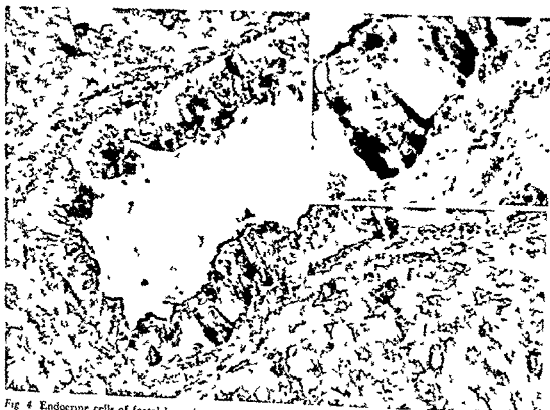


Fig 4 Endocrine cells of foetal lung (case number 16) stained by Grimelius silver nitrate stain × 1800 insert × 4500

low cone which might or might not reach epithelial surface (Fig 7 8 9 and 10) The fluorescent material sometimes appeared to be confined to very small granules in the cytoplasm

In the absence of L-DOPA some cells emitted a greenish fluorescence of varying intensity often very faint probably showing the presence of varying amounts of dopamine (Figs 11 12 13 and 14) The approximate number of these cells corresponded to the number of cells blackened by the silver technique of Davenport

If formaldehyde gas was omitted from the histochemical procedure fluorescence did not appear Fluorescence of both incubated and non incubated cells faded rapidly when exposed to irradiation with ultra violet light especially the very faintly fluorescent cells in specimens not (L-DOPA) incubated

Fig 1 and 2 Endocrine cells of foetal lungs (case number 20 and 8) stained by HCl toluidine blue Fig 1 0 2N HCl 60° C 11 hr toluidine blue pH 3 2 × 1400

Fig 2 0 2N HCl 60° C 9 hr toluidine blue pH 4,8 × 875

Fig 3 Endocrine cell of foetal lung (case number 4) stained by Pb-H × 1800

DISCUSSION

A common function of APUD cells is probably secretion of low molecular weight polypeptide hormones which are believed to be stored in secretory granules *Pearse* (1969 b) proposed that the polypeptide hormones of the APUD series of en-docrine cells are synthesized and stored as protein precursors with a primary structure which includes a high proportion of carboxyl and carboxamide side



Fig 5 Endocrine cells of foetal lungs (case number 24) stained by Grimelius silver nitrate stain × 3500

Fig 6 Endocrine cells of foetal lung (case number 16) stained by Davenport's silver nitrate stain × 1800

Fig 7 8 9 and 10 Fluorescence photomicrographs of foetal lungs (case number 34 and 36) Formaldehyde reaction of specimens incubated with L DOPA × 875 (7 9) × 1400 (10)


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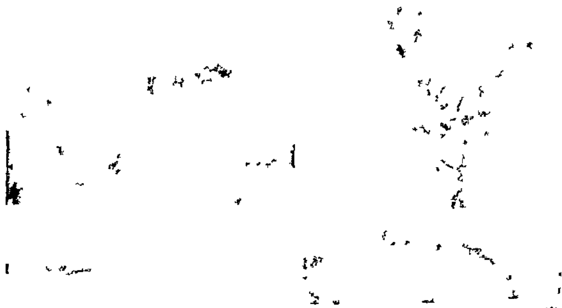

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Figs 11 12 13 and 14 Fluorescence photomicrographs of foetal lungs (case number 31 and 36) Formaldehyde reaction of specimens not incubated × 875 (11 12) × 1400 (13 14)

chains and a secondary structure which is predominantly that of a random coil polymer the latter because metachromatic staining with HCl toluidine blue technique primarily seems to indicate a random coil conformation

Similarity between Pb H and HCl toluidine blue as stains for endocrine cells was discussed by *Solcia et al* (1969 b) As a rule cells stained by Pb H were also reactive to the HCl toluidine blue technique In my hand staining by Pb H detects fewer cells than does HCl toluidine blue

Silver impregnation does not delineate specific chemical groupings and the histochemical significance of argyrophilia of endocrine cells is unknown Most cells storing biogenic amines are both argentaffin and argyrophil The argentaffin reaction is believed to show a high concentration of reducing agents such as catecholamines and 5 HT Amine, when present probably accounts at least in part for the argyrophilia (*Solcia et al* 1969 b) but many argyrophilic cells do not contain biogenic amines although they take up amine precursors Another characteristic feature of these cells is a secretory product which is stored in membrane bound granules *Cavalheira et al* (1968) have pointed out the possibility that the constitution of this membrane may be responsible for the argyrophilic reaction

In the paper by Grimelius as well as in that by Hellman and Hellerstrom it was pointed out that some grade of autolysis is necessary for optimal staining reaction My material was fixed in ice cold fixatives and the fixation *per se* was carried out at 0–4° C In the present study, argyrophilic cells to be detected were more numerous than cells reactive to staining with HCl toluidine blue and Pb H This might be due to a low number of granules in the cells as commonly is the case of G-cells of the pyloric mucosa (*Pearse et al* 1970)

The histochemical fluorescence method of Falck and Hillarp (*Falck et al* 1962 1965) for demonstration of biogenic mono amines is based on the finding that amines can be condensed with formaldehyde to yield strongly fluorescent compounds provided that they are enclosed in a dried protein layer, as in freeze dried or air dried tissues This method is superior to all other histochemical methods for the demonstration of biogenic mono amines

By this method mono amines were only demonstrated in few of the bronchial cells However a large number of fluorescent cells were demonstrated in L DOPA incubated specimens indicating that amine mechanisms do operate in these cells The amine handling property is a common feature of APUD cells but any explanation of the part played by amines in synthesis storage or secretion of the polypeptide hormone product, has not been developed

In foetal lungs and in lungs of children, *Feyrter* demonstrated some of the paracrine cells to be argyrophilic In lungs of adult human subjects none of the paracrine cells were argyrophilic Perhaps these cells have the properties of taking up amine precursors and thereby the possibility of synthesis storage or secretion of a polypeptide hormone product It is known that oat cell carcinoma of the lung occasionally produces an ACTH like substance and carcinoid tumours sometimes give rise to carcinoid syndrome *Feyrter* (1954 1960) suggested that carcinoid tumours might arise from paracrine cells in the bronchial epithelium In carcinoid tumours he demonstrated argyrophilic as well as argentaffin cells

CONCLUSION

In the bronchial epithelium of foetal lungs cells which were believed to be members of the APUD series of endocrine cells were demonstrated *Some* cells were stained by HCl toluidine blue and Pb H suggesting a cytoplasmic protein of random coil conformation *Some* argyrophilic but non argentaffin cells emitted a greenish fluorescence of vayring intensity by the formaldehyde condensation method Only few of these cells were reactive to staining with HCl toluidine blue and Pb H suggesting that they might be poorly granula

ted *Some* cells were only visible after incubation with L-DOPA emitting a green formaldehyde-induced fluorescence, probably reflecting the presence of dopamine formed intracellulary from the administrated L-DOPA These cells might be completely degranulated, but perhaps they represent undifferentiated cells having the properties of synthesis, storage or secretion of polypeptide hormone, because of their amine-handling properties

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I am indepted to professor *K Kristoffersen* M D, chief gynaecologist, and to *J Jacobsen* M D for the foetuses kindly placed at my disposal

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Acta path microbiol scand Section A *80*, 235–240, 1972

A CASE OF THYMOMA IN ASSOCIATION WITH ERYTHROCYTOSIS

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A case of erythrocytosis and coexistent thymoma is presented The erythrocytosis was diagnosed about two years before the patient's death The thymoma was found at autopsy The relationship between the two disorders is discussed

Thymoma and dysplasia of the thymus have been observed in association with a number of extra-thymic diseases of which bone marrow hypoplasia with pure red cell agenesia (PRCA) is common A review concerning these observations has recently been published by *Goldstein & Mackay* (1969)

It seemed to be of interest to report the following patient *with erythrocytosis and concurrent thymoma* As far as is known, no association between these two diseases has been described in detail previously In a work of *Friedman* (1967) a case of polycythaemia and thymocarcinoma is *mentioned* (in Table II) without further comment

CASE REPORT

Clinical Data

A man (F J, 890624), 78 years old, sought medical advice for a dry throat and a moderate cough He had been essentially healthy up to that time He was found to have a haematocrit value of 74 per cent He had lost about 10 kg in weight during the last three years and in the last year been troubled by some dyspnoea on effort During the last three months he had noticed swelling of the lower legs

Positive clinical findings on admission Moderate cyanosis of the lips, no dyspnoea at rest, and pretibial oedema Weight 57 9 kg The lingual mucosa atrophic and reddened Blood presure 150/90 mm Hg (recumbent position) Slight dullness of the left side of the chest on percussion The liver was palpable 2 3 fingers below the costal arch

Laboratory data on admission Hb 20 4 g/100 ml, haematocrit 72 per cent The white blood cell count lay between 8 200 and 15 300/mm³ with normal differential count The thrombocyte count varied between 89 000 and 373 000/mm³ Sedimentation rate 0 mm/h The reticulocytes varied between 0 4 and 4 8 per cent Sternal punctate showed at this time an abundance of fat, the presence of plasma cells, and normal megacaryocytes, indicating secondary polycythaemia On the other hand, phosphatase staining of granulocytes in blood smears

(calculated normal value 4 7 l) (the alveolar carbon monoxid method according to Sjostrand) The serum iron varied between 18 and 65 mg/100 ml, TIBC was 306 mg/100 ml Total protein in serum 7 g/100 ml

Treatment and Course

Clinically the diagnosis polycythaemia vera was settled and the patient initially was treated with repeated venesections (total 2 700 ml) and both a subjective and objective (Hb 18,2 g/100 ml, haematocrit 65 per cent) improvement was obtained However, the blood values could only be kept under control temporarily by this means and treatment with radioactive phosphorus (^{32}P) was initiated A total of 16 2 mC was given but gave only temporary improvement Some small venesections (total 350 ml)

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Fig 1 a b X rays taken 6 months before the autopsy, showing a round mediastinal tumour near the peri cardium



Fig 2 Macrophotograph showing the thymoma found at autopsy The tumour was situated in front of the tracheal bifurcation and was supplied with a fibrous capsule A haemorrhage was found in the interior of the tumour

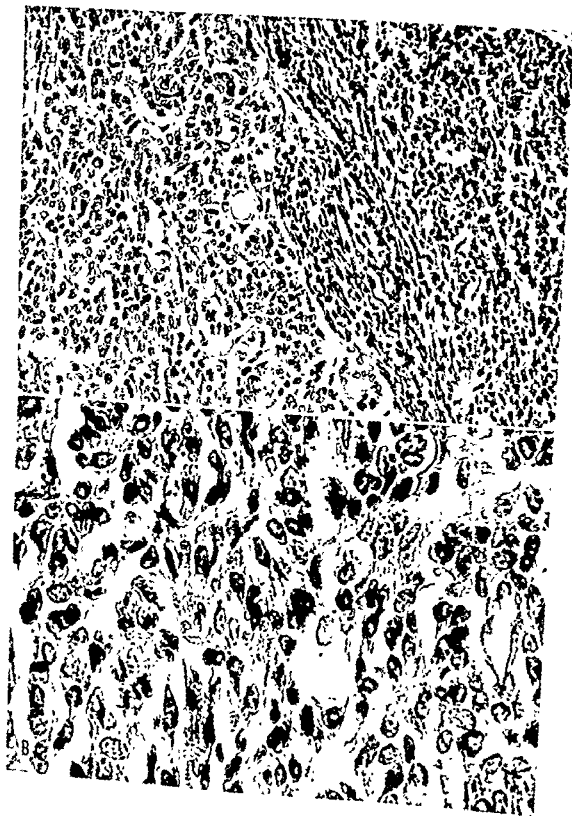
were tried Sternal puncture now showed a mode rately hyperplastic bone marrow with intensive erythropoiesis and the presence of cells from the myeloid series megacarocytes and fat cells Radioactive phosphorus (6 mC) was given, 7 months after the previons dose and some small venesect (total 400 ml) were performed About one mo later the patient s condition deteriorated his fat became more pronounced and he was confined bed (Hb 18 6 g/100 ml)

Pulmonary roentgenography now revealed round tumour in the central anterior part of mediastinum in the immed ate vicinity of the terior thoracic wall (size 10 × 9 cm in the fro projection) (Fig 1) Needle biopsy of the tum was considered to be compatible with a neuroge tumour (neurinoma neurilemoma)

Busulphan therapy was now given The patie condition improved but two weeks later the bl values had again increased (Hb 18 9 g/100 m Venesection was performed (200 ml) Three we later there was an acute deterioration with hi

Fig 3 a Microphotograph of a specimen from t thymoma The tumour has a epithel al structu note its mixed appearance To the left the ce are more clearly epithelial and to the right cells the spindle like type lying in bundles can be see According to the terminology suggested by Renau (1968), among others this is considered to r present a lympho-epithelioma with mixed epithe lial predominance (× 180, v G)

Fig 3 b Microphotograph of the same specimen a higher magnification Note the epithelial structure (× 540 v G)



fever, dyspnoea, tachycardia, and rales over the lung bases Despite parenteral penicillin therapy the patient died on the following day

Autopsy Findings

Autopsy was performed less than 24 hours after death The heart (weight 350 g) showed no signs of fresh infarction In the aorta pronounced athero matous changes were seen In the pulmonary ar teries there were some small fresh emboli bilaterally In the lower lobe of the left lung considerable oedema and yellowish white broncho pneumonias were found The spleen was enlarged (260 g), soft and friable The kidneys exhibited slight nephro sclerotic changes (2 × 130 g) In the liver (1 370 g) signs of haemostasis In the basal part of the left temporal lobe of the brain there was a small infarction (weight of brain 1 230 g)

In the mediastinum a tumour the size of a tennis ball was found, located in front of the trachea at the level of the bifurcation The tumour was well defined and supplied with a fibrous capsule It was slightly adherent to the mediastinal surface of the left lung From the upper pole of the tumour there was a small vascular communication with the thyroid region The cut surface of the tumour showed a large central haemorrhage No metastases were found (Fig 2)

Sections from the tumour were stained according to van Gieson, haematoxylin eosin periodic acid Schiff s reagent (PAS), at methyl green pyronine The viable outer region showed close to the capsule a fairly homogeneous tumour tissue with spindle shaped cells arranged in strands and whorls No palisade arrangement, marked anisocytosis or in filtration of the fibrous capsule or surrounding tissues were observed Towards the centre of the tumour the tissue consisted of alternately oval and polyhedral cells with loosely arranged chromatin and pale cytoplasm, arranged fairly irregularly sometimes in whorls and strands The interstices were filled in some places with slightly granular material which stained weakly with eosin and strongly with PAS The cytoplasm was weakly positive to eosin and negative to PAS or contained PAS positive granules in some places Small aggre gates of lymphocyte like cells were observed close to the capsule and in small fibrous septa in the tumour There were no germinal centres and only a sparse occurence of pyronine positive plasma cells (Fig 3 a b) The histopathological picture was considered to be well compatible with a thymoma a lympho-epithelioma of predominantly mixed epi thelial type

DISCUSSION

It was evident from the findings presented above that this patient suffered from two well defined and distinguishable diseases, one of which was diagnosed clinically as poly cythaemia vera and the other at autopsy as thymoma

The patient showed no clinical or patho-anatomical signs of any diseases which are commonly associated with secondary poly cythaemia, *i e* chronic pulmonary disease renal disease The ventilatory capacity was reduced, but this was not considered to be of any clinical importance (*Sodeman & Sode man* 1967) Although the patient clinically was treated as a polycythemia vera several clinical and laboratory data contradicted this diagnosis essentially normal bone marrow activity in sternal punctate at onset of disease absence of trombocytosis (*Sodeman & Sode man* 1967)

The tumour found at autopsy was localized to the anterior mediastinum close to the an terio superior surface of the pericardium It was well encapsulated and showed no invasive growth into surrounding tissues Microscopi cally, the tumour consisted of epithelial ele ments with somewhat varying arrangements From the patho anatomical evidence ob tained it was diagnosed as a benign thymo-ma, a lympho epithelioma of predominantly mixed epithelial type (*Renault* 1968 *Galy & Renault* 1969, *Goldstein & Mackay* 1969)

As regards polycythaemia vera and lym pho epithelioma of the thymus, little is known about their aetiology and pathogenensis A discussion concerning the association between these two diseases can therefore of necessity only suggest possible explanations

1 One may, like *Dameshek* (1951) and *Wasserman* (1954), regard polycythaemia vera as a neoplastic process, a variant of the myeloproliferative syndrome The polycythae-mia could then possibly be interpreted as a manifestation of a defect in the cellular im munity due to a previously developed thy moma causing an immunological tolerance towards the in some way, neoplastic bone marrow cells

2 *Jepson & Lowenstein* (1966) observed the presence of inhibitors of erythropoietin in plasma from two patients with PRCA one of

whom had a thymic tumour *Barnes* (1965) discovered an antinuclear antibody in sera from patients with thymoma and refractory anaemia *Barnes & Xefteris* (1966) showed, further that these sera were capable of inhibiting the proliferation and maturation of normal bone marrow cells *in vitro* *Field et al* (1968) studied the marrow suppressing effect of serum from a patient with thymoma, PRCA and Hodgkin's disease It was suggested that an erythropoiesis inhibiting factor might have been produced in excess as a result of the thymoma They considered that this factor probably acted directly on the bone marrow and not via inhibition of the erythropoietin production If such a factor is normally of importance for physiological control of erythropoiesis, possible absence of this factor in a thymoma of a different type might result in erythrocytosis

3 It has been shown in several studies (*Law* 1952 *Law et al* 1950, *Miller* 1962) that the thymus has a central role in leukaemogenesis in certain mouse strains This may possibly take place via the lymphocytosis stimulating factor (LSF) described by *Metcalf* (1956), among other authors *Andersen & Pedersen* (1967) reported three cases of acute leukaemia with a concurrent thymoma The authors discussed the possibility that the thymus plays a primary role in leukaemogenesis in man But no indications of this kind have yet been found, and still less that such a mechanism could explain the simultaneous occurence of erythrocytosis and thymoma in the present case Such a possibility cannot be excluded however Polycythaemia has been observed in association with renal and extra renal tumours, possibly secreting an erythropoiesis stimulating factor (*Sodeman & Sodeman* 1967, *Jepson* 1969)

According to a classification suggested by *Jepson* (1969) the major defect in primary polycythaemia (vera) seems to be in the bone marrow, independent of known humoral stimulation (erythropoietin, ESF) The secondary polycythaemia is considered to be "a manifestation of an extra medullary lesion" and appears to be humoral-dependent

Whether the erythrocytosis in the presented case is of primary or secondary type cannot be determined with any certainty There are certain facts contradicting the diagnosis polycythaemia vera, as mentioned above In the hypotheses (1–2) discussed above the thymoma is assumed to be primary and the development of erythrocytosis a secondary event These explanations contain no assumption of any raised erythropoietin production Thus, according to *Jepson* (1969), the erythrocytosis would be of polycythaemia vera-type According to the last hypothesis (3) production of erythropoietin in the thymic tumour causes polycythaemia, this time of secondary type No information elucidating the aetiology of the thymoma *per se* has been obtained The observed association between thymoma and erythrocytosis in the present case seems though to confirm the earlier known association between thymic and disturbed erythropoiesis

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LOCATION OF RENIN IN RABBIT UTERUS BY HELP OF MICRODISSECTION

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The location of renin was investigated on microdissected cryostat section of freeze dried uterine tissue from three *non pregnant* and six 28 days *pregnant rabbits* A coarse separation of the uterine layers of the *mesometrial* part of the wall showed about the same high renin content in the endometrium and the circular myometrium but a much lower content in the longitudinal myometrium In the *antimesometrial* part of the wall the renin content was lower in the endometrium than in the two muscle layers In the pregnant *endometrium* renin was mainly located to the endometrial stroma in which high values were measured in smaller vessels (40-60 μ in diameter) In the pregnant *myometrium* renin was located both to the arteries arterioles and venoles, the renin concentration being especially high in the smaller vessels (30 60 μ), the values of the venoles exceeding those of the arterioles In addition to the isolated vessels renin was found in muscle and connective tissue devoid of arterioles and venoles This can be due either to ubiquitous occurrence of renin producing cells, or to release of renin from the vessels to the extracellular fluid

In a previous paper (*Eskildsen* 1971) the formation of renin in the rabbit uterus was investigated by autotransplantation of pieces of endometrium and myometrium to the anterior chamber of the eye The study showed that grafts of endometrium as well as of myometrium synthezised considerable amounts of renin in the course of 7 to 14 days Subsequently it seems natural to concentrate the further investigations of the location of renin formation on the vessel walls, as they are common for the two, from a morphological point of view, rather unlike uterine layers For this purpose the vessels in the rabbit uterus were isolated by microdissection of freeze dried tissue (*Faarup* 1967 & 1968) and subsequent estimation of renin in the isolated structures The paper includes the results of a coarse microdissection of uteri from normal, non pregnant rabbits, and a more detailed dissection of uterine tissue from pregnant animals just before delivery At this point the uterine renin content reaches its summit twenty to hundred times higher than in the non pregnant uterus (*Bing* & *Faarup* 1966)

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MATERIALS AND METHODS

Nine female albino country rabbits the State Seruminstitute weighing 3 to 4 kg were used, 3 of them being non pregnant and 6 pregnant at the 28th day of pregnancy During anaesthesia with pentobarbital sodium intravenously (40 mg/kg body weight) segments of the uterine horn were removed quickly and immediately frozen in a mixture of isopentane and dry ice (–65°C) In one non pregnant rabbit and two pregnant the uterus was frozen *in situ* by dipping one horn in a bath

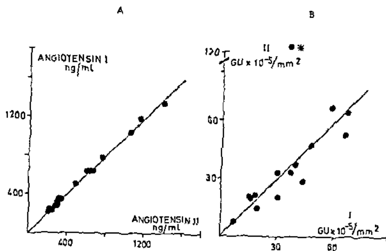


Fig 1A The concentration of angiotensinogen, expressed in ng angiotensin per ml plasma estimated in 18 plasma samples by *radioimmunoassay* for angiotensin II (the abscisse) as well as by radioimmunoassay for angiotensin I (the ordinate)

B The *renin concentration, expressed in Goldblatt* units × 10^{-5}/mm² of the freeze dried tissue in 15 pairs of uterine preparations, one horn being *frozen in situ* (I – the abscissa) before removal of the tissue while tissue from the other horn was frozen *after* removal (II – the ordinate) Only in one case (marked *) the results showed, for unknown reasons no agreement

of isopentane cooled by liquid nitrogen (–160°C), after which a segment of the frozen uterus was cut free The frozen tissue was cut in a Pearse Slee Cryostate (–20°C) in 20 micron thick sections arranged in series Excication was performed in petri disks containing phosphorus pentoxide, P_2O_5, the *first twenty four hours at 20°C, then at room temperature* in an excicator In the non pregnant animals cross sections were cut of a whole uterine segment, while in the pregnant animals, where the diameter of the horns is ten to twenty times enlarged, only smaller parts of the mesometrial, lateral and antimesometrial zones of the uterine wall were cut in the cryostat

scc

nually with pieces of razor blades (Blue Gilette) fixed in a small artery forceps (*Faarup* 1967) The precision using this technique is about 10 to 20 micron During the microdissesection the freeze dried sections were placed on a slide and covered with paraffine oil (Ph Nord) which inhibited water absorption and cleared the tissue, making the structures visible The microdissected preparations were isolated from 3 20 sections cut in series In consequence of varied thickness from section to section ±25% the different comparative preparations were isolated from the same sections and in almost the same amount from every section

Area determination used for quantitation of the microdissected preparations was performed by planimetry of photoes (Reichert Automatic Camera) of every isolated structure, and expressed in mm² In case of isolated vessels and tissue containing vessels, the area of the lumens was subtracted

Tissue extraction – The microdissected tissue fragments were transferred from the slides with fine insect nails to homogenisator pistles homogenized for 3 minutes at 4°C in 350 μl 0 2 M tris/HCl buffer, pH 7 5 placed for one to two hours at 4°C and thereafter centrifuged

Renin assay combined with radioimmunoassay for angiotensin I was based on the principle of determination of the decrease in angiotensinogen concentration in course of time (*Poulsen* 1965b) 75 μl tissue extract, or dilutions of this (using 0 2 M tris/HC buffer pH 7 5) were incubated for 24 or 48 hours at 37°C with 25 μl of a renin substrate a pool of 24 hours nephrectomized rat plasma, containing a fixed concentration of angiotensinogen (ca 2000 ng/ml) Transformation of the angiotensinogen to angiotensin I was performed during incubation for 10 minutes at 37°C with excess of a purified hog renin preparation (Nutritional Biochemicals – Cleveland) used in a concentration of 1 Goldblatt unit per ml (diluted with 0 2 M tris/HCl buffer pH 7 5) and added 3 mM EDTA (*Haber et al* 1969) The concentration of angiotensin I was measured by means of radioimmunoassay for angiotensin I carried out as described previously for angiotensin II (*Poulsen* ... (1 Asp ... weighs ... bioassay ... ensin II





Fig ...ained section, 20 micron (μ) thick, of a non pregnant uterine horn showing the mesometrial (MES), the two lateral (LAT) and the antimesometrial (ANTIMES) zones Magnification 15 X. This preparation is, like the following embedded in parafine oil
B A segment of the mesometrial wall of the non pregnant uterus demonstrating the sharp separation between the endometrium (E), the circular myometrium (MC) and the longitudinal myometrium (ML) with the interjacent connective tissue (CT) containing the large vessels In the endometrial fold, distinction between the epithelial fraction (EP), the peripheral (P) and the basal (B) part of the stroma is marked by dotted lines Magnification 30 X

after transformation to angiotensin II In several plasma samples (n = 18) from rats the angiotensinogen concentration was measured by the earlier used radioimmunoassay for angiotensin II as well as by the new modification of radioimmunoassay for angiotensin I, which resulted in equal values (Fig 1A) The reproducibility of the assay was estimated by repeated assays of the angiotensinogen concentration of the same plasma sample, which showed a S D of ± 5 % (n = 15) including the counting inaccuracy (S D ± 1 %)

Renin concentration in the extracts of microdissected preparations was expressed in Goldblatt units per ml (GU/ml) by reference to a highly purified hog renin standard preparation*), which in a series of dilutions $5 \times 10^{-5} - 10 \times 10^{-5}$ GU/ml was included in every assay The renin concentration of the microdissected preparations was further corrected to the area and, since the extracting volume was constant (350 μl), expressed in GU $\times 10^{-5}$/mm²

Control of the influence of the paraffine oil and the dissection procedure on the renin content in the uterine tissue – Identical preparations of freeze dried uterine tissue (sections in series of pregnant uterus) were placed in an exsicator for 0 1 2 4 and 8 days using for each period two samples one in oil, one dry Renin determination showed that the treatment with oil did not produce any change in the renin content

A possible *loss* of renin during the microdissection was controlled with a *fine dissection* on series of identical preparations every second being cut into small pieces (area in average 0 5 – 0 1 mm²), the others remaining intact In the case of tissue frozen *after removal* the dissection resulted in a loss of about 30 % whereas the loss only was 10 % in case of tissue frozen *in situ* On the other hand when the tissue was exposed to a *coarser* dissection

*) Kindly supplied by dr *Haas*, and identical with the preparation donated by dr *Haas* to the WHO Laboratory for Biological Standards (Nat Inst Med Res Mill Hill, London)

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Fig 3A The contracted wall of the one pregnant uterine horn frozen after removal of the tissue
B The thin dilated wall of the other horn frozen *in situ* Both sections belong to the mesometrial zone Magnification 15 X
C The uterine wall frozen *in situ* further magnificated to distinguish the different uterine layers also demonstrated in 3A (the abbreviations correspond with those used in Fig 2B) Magnification 30 X

(area of the pieces in average 1 mm²) no significant loss of renin was detected In this case the two kinds of freezing technique showed concordant values (Fig 1 B)

RESULTS

1 Distribution of Renin in the Uterine Wall

In two non pregnant and four pregnant animals the uterine wall was separated in the endometrium and the myometrium which was further divided in the central circular layer and the peripheral, longitudinal layer This separation was performed on tissue from the mesometrial the lateral and the anti mesometrial zones (Fig 2A)

Comparing the *non pregnant* with the *pregnant* animals markedly higher values were seen in all three layers of the pregnant animals (Fig 4)

In consequence of the great individual variations it was very difficult to see any difference in concentrations of renin in the *endometrium* and the *circular* myometrium but the values of the *longitudinal myometrium* were as a whole lower than those of the endometrium and the circular myometrium

In the pregnant animals the renin concentration of the endometrium and the circular myometrium tended to decrease from the *mesometrial* to the *lateral* zone and further to the *antimesometrial* (in a single animal however, the values were for unknown reasons increasing towards the lateral zone) In the longitudinal myometrium the values were higher in the antimesometrial than in the mesometrial zone

2 Renin Concentration in Epithelium and in Stroma of the Endometrium

In order to study whether there is a correlation between the vessels especially the ar

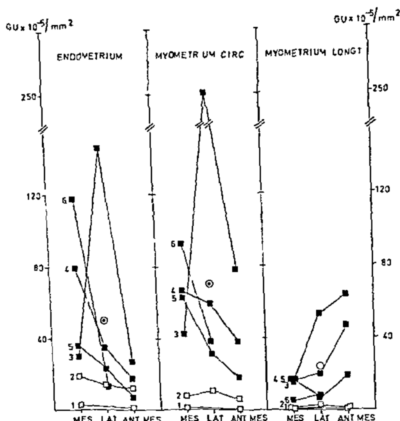


Fig 4 The distribution of renin GU × 10^{-5}/mm between the endometrium the circular and the longitudinal myometrium in 2 non pregnant (□) and 4 pregnant animals (■) Concerning the pregnant animals the mean value of each layer is shown (○) In the endometrium as well as in the myometrium distinction is made between tissue isolated from the mesometrial (MES) the lateral (LAT) and the antimesometrial (ANTIMES) zone The numbers refer to the animal numbers

terioles and the renin content in the endometrium of the *non pregnant* uterus a mesometrial fold of the endometrium from three animals was divided into three fractions according to the distribution of vessels (Fig 2B) 1) a basal and central part of the endometrial stroma containing the larger vessels especially arterioles 2) a peripheral part of the stroma containing ramifications from the central larger vessels and finally 3) a superficial epithelial fraction containing besides the surface and glandular epithelium zone subepithelial capillaries The renin concentration (Fig 5) was lower in the epithelium than in the stroma which showed only smaller differences between the peripheral and the basal part even though the results tended at a higher concentration in the basal fraction

In the pregnant uterus the endometrium (Fig 3) in consequence of the violent dilatation af the horn is a thin flattened layer covering the much thicker myometrium which makes any division of the endometrial stroma impossible with the present method The high renin content of the pregnant uterus however made it possible to measure the renin content even in only a few isolated vessels In six cases a comparison was made between 1) the epithelium of the surface and the glands and 2) isolated vessels from the endometrium of the mesometrial and lateral zones (Fig 6) The isolated vessels mainly arterioles but also some venoles were dis-

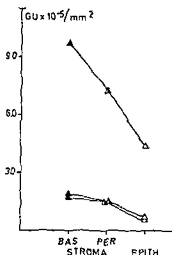


Fig 5 Distinction between the renin concentration GU $\times$ 10 ^{5}/mm², in the basal part of the endometrial stroma (▲), the peripheral part of the stroma (*Δ*) and the surface and glandular epithelium (Δ), *separated by microdissection of an* endometrial fold in the mesometrial zone of 3 non pregnant animals

sected as single vessels and as groups of vessels with thin strands of the surrounding endometrial stroma The renin concentration is seen to be about 7 times higher in the vessels than in the epithelium This agrees with the findings in the non pregnant animals (Fig 5) showing that the renin in the endometrium is mainly located to the vessels or the stroma cells surrounding them, while the much smaller renin content of the epithelium may be caused by the small amounts of stroma included in these preparations

3 Renin Concentration in Vessels isolated from Myometrium

The rich blood supply to the pregnant uterus is characterized by the content of several arteries and veins (150–300 μ in internal diameter) in the thick layer of connective tissue between the two muscle layers, supplied from the large mesometrial vessels (about 300 μ in diameter) During their course towards the antimesometrial zone they give off, centrally plenty of arterioles and venoles (75 150 μ in diameter) which are ramificated further in smaller branches (30–60 μ in diameter) during the penetration of the circular muscle layer towards the endometrium Only smaller arterioles, venoles and capillaries are found peripherally in the longitudinal layer of the myometrium and the serosa Finally, the vessels between the muscle layers are supplying two pairs of aa and vv uteri laterales (ca 300 μ in diameter), running along the uterine horn on each site

In the circular layer of the myometrium vessels were isolated and divided into groups with internal diameters of 30–60 μ 75–150 μ and 150–300 μ, isolated from the same freeze dried sections (Fig 7 and Fig 8A) The isolated vessels were predominantly arteries and arterioles (characterized by two or several concentric layers of muscle cells), but in the unstained sections it was sometimes difficult in the case of 30–60 μ vessels to decide with certainty whether an isolated vessel was an arteriole or a venole It is seen in Fig 8A that the tendency is towards a decrease in the renin content when the diameter is increased In three preparations of vessels isolated from tissue frozen *in situ* this tendency was, however, not so marked and in one case the highest value, 60 $\times$ 10 ^{5} GU/mm², was even measured in the group of 150–300 μ vessels As the freezing technique kept the vessels, like the entire uterine horn, dilated it was almost impossible in this type of tissue to distinguish arteries and arterioles from veins and venoles

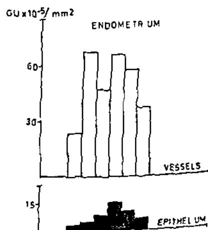


Fig 6 The renin concentration *GU* $\times$ *10* ^{5}/mm² in isolated surface and glandular epithelium compared with that of isolated single vessels or groups of vessels, from 6 preparations *of endometrium* from 4 pregnant animals

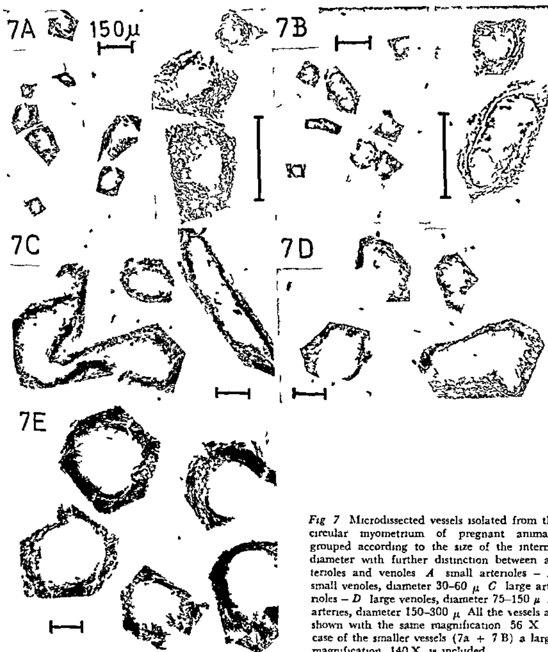


Fig 7 Microdissected vessels isolated from the circular myometrium of pregnant animals, grouped according to the size of the internal diameter with further distinction between arterioles and venoles *A* small arterioles – *B* small venoles, diameter 30–60 μ *C* large arterioles – *D* large venoles, diameter 75–150 μ *E* arteries, diameter 150–300 μ All the vessels are shown with the same magnification 56 X In case of the smaller vessels (7a + 7 B) a larger magnification 140 X is included

In three cases, preparations of 1) typical arterioles and 2) typical venoles were isolated and divided into two groups, with a diameter of 30–60 μ another with a diameter of 75–150 μ (Fig 7, Fig 8B) In all cases, the renin values of the venoles exceeded those of the arterioles, this being particularly pronounced in the case of the small vessels Compared with Fig 8A, the values in Fig 8B seem low, which is probably due the fact, that they derived from different animals as there was a similar difference between their total uterine renin content

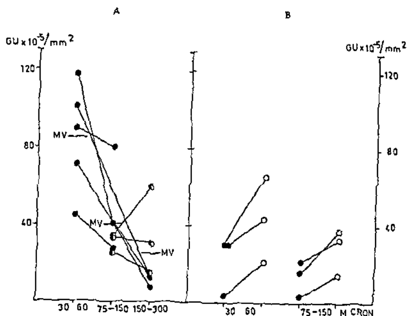


Fig 8A The renin concentration GU × 10⁻⁵/mm² in myometrial vessels mainly arterioles (●) grouped according to the size of the internal diameter (30–60 μ 75 150 μ and 150 300 μ) In the cases of uterine tissue frozen *in situ* the isolated vessels were a mixture of arterioles and venoles (◐) The horizontal lines indicate the mean value (M V) *B* Comparison between the renin concentration of typical arterioles (●) and typical venoles (○) with diameters of 30 60 μ and 75–150 μ isolated from three preparations of circular myometrium different from those used in 8A

4 Renin Concentration in Muscle and Connective Tissue with Different Vascularization

From the previous results it appeared that the renin content in the vessels of the circular part of the myometrium was especially high in the smaller vessels and higher in the venoles than in the arterioles Comparing the values of the whole circular myometrium (*Fig 4*) with those of the isolated vessels (Fig 8) it was remarkable that only in few cases the renin concentration of the isolated vessels exceeded that of the whole myometrium As the about 30 per cent loss of renin during the detailed microdissection (see Materials and Methods) can not explain this finding renin producing cells must be located in high consentrations in places other than the isolated vessels A possible source was the tiny venols (<30 μ in diameter) and the capillaries which could not be isolated manually In several cases a comparison was therefore performed between 1) muscle tissue containing plenty of tiny vessels venoles and capillaries but without any arterioles (Fig 9A) and 2) muscle tissue almost without any vessels other than capillaries (Fig 9B) It was demonstrated that the renin concentration of the well vascularized muscle tissue was about 3 times higher than that found in the tissue devoid of arteriols and venoles (*Fig 11*) In spite of this marked difference it was remarkable that muscle tissue almost without any visible vessels (magnification × 100) contained considerable amounts of renin

In order to see whether the distribution of vessels and not the content of surrounding connective tissue included in the isolated vessels, might be the decisive factor a comparison was made between 1) strands of connective tissue from the circular myometrium containing plenty of capillaries and venoles (Fig 10A) and 2) strands containing very few of these vessels (Fig 10B) The results of this study (*Fig 11*) further established that the content of tiny vessels and not the connective



Fig 9A Muscle tissue isolated from the circular myometrium of the pregnant uterus containing smaller venoles (<30 μ in diameter) and capillaries *B* Muscle tissue without visible venoles and capillaries Magnification 140 X

Fig 10A Strokes of connective tissue isolated from the circular myometrium of the pregnant uterus containing smaller venoles (<30 μ in diameter) and capillaries

B Connective tissue without visible venoles and capillaries Magnification 140 X

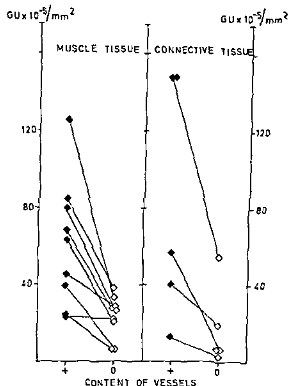


Fig 11 The renin concentration $GU \times 10^{-5}/mm^2$, in muscle tissue and connective tissue with several small venoles (+ — ◆) and without visible venoles (O — ◇) isolated from the circular myometrium of 5 pregnant animals None of the preparations contained arterioles

tissue was important for the high content of renin in the tissue, although the connective tissue devoid of these vessels contained renin

DISCUSSION

From the demonstrated influence of oestradiol on the renin content both in endometrium and myometrium and the observed production of renin in intraocular autografts of endometrial and myometial tissue (*Eskildsen* 1970 and 1971) it is shown that the renin formation in rabbit uterus takes place in the endometrium as well as in the myometrium Subsequently the present attempt to localize the uterine renin was turned towards the vessels distributed to both uterine layers Furthermore location in the uterine vessels would be in agreement with previous findings of considerable content of renin in different vessel walls, for instance in the hog aorta and hog vein (*Gould et al* 1964) and in the smaller mesenteric arteries in dogs (*Genest et al 1968*) Previous studies have further shown that renin in the kidney of different mammals and in the submaxillary glands of mice is localized to distinct morphological structures As regards the kidney several investigators (*Hartroft et al* 1964 *Faarup* 1967 & 1968, *Cook* 1971) have demonstrated that the renin is exclusively localized to the afferent vessel of the juxtaglomerular apparatus In the submaxillary glands the very high amounts of renin were found in the striated ducts (*Bing & Faarup* 1965)

The results of the present study however showed that, even though distinct differences in renin concentrations were measured in the microdissected preparations no isolated tissue fractions of pregnant uterus were devoid of renin As described in a previous microdissection study (*Bing & Faarup* 1966) the renin of the pregnant uterus was found to be distributed in high concentrations to all layers and parts of the uterus although smaller changes between the mesometrial lateral and antimesometrial zones in the endometrium and the myometrium were seen The present detailed microdissection of pregnant uteri further showed that renin was present in vessels of all sizes both arterioles and venoles as well as in different types of tissue without these vessels

Comparison (Table 1) of the mean values of these results (calculated to GU/g wet tissue) with the average concentrations of renin in kidney, non pregnant uterus ureter and plasma emphasize the high level of even the lowest measured values of the microdissected uterine tissue

The distribution of renin to all isolated structures of the uterine wall must be explained either by a similar wide distribution of renin producing cells or by an occurrence of rather large amounts of renin in the extracellular fluid released from the vessels

This work was supported by grants from *Nordisk Insulinfond* and *The Foundation of P Carl Petersen*

TABLE 1. Average Concentrations of Renin in Different Rabbit Tissues

A	Organs		GU/g
	Kidney		20
	Pregnant uterus		16
	Non pregnant uterus		1
	Ureter		10^{-3}
	Plasma		10^{-4}
B Animal no	Microdissected tissue from pregnant uterus		GU/g
	Endometrium		
3–6	Vessels		11
	Epithelium		2
	Myometrium	30–60 μ	23
5–8	Vessels	75–150 μ	10
	(Chiefly arterioles)	150–300 μ	7
3–4	Arterioles	30–150 μ	5
	Venoles	30–150 μ	9
3–8	Muscle tissue and	several tiny vessels	18
	connective tissue containing	few tiny vessels	5

The renin concentration mean values, of different organs compared with the concentrations of the microdissected vessels and tissue fractions from six pregnant uteri (animal no 3–8) The mean values of the freeze dried isolated preparations are converted to GU/g on the background of the ratio between the measured values of wet tissue (16 GU/g) and freeze-dried tissue (60×10^{-5}/mm²) of pregnant uterus. The microdissection was performed on tissue from rabbits with individual difference in uterine renin content, explaining the lower values of isolated vessels from animal 3 and 4

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Acta path microbiol scand Section A 80, 253-256, 1972

INFLAMMATORY CELLULAR REACTION IN HYPERTENSIVE VASCULAR DISEASE IN MAN

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It is demonstrated that an inflammatory mononuclear cellular infiltration into hypertensively damaged arterioles and small arteries takes place in man, independent of the aetiology of the systemic hypertension This cellular infiltration was observed both in vessels in which marked degenerative changes were recognized and in vessels in which no degenerative changes could be observed under the light microscope The mononuclear cellular infiltration is possibly due to a hypersensitivity of the delayed type

Acute experimental hypertension caused by repeated intravenous injections of angiotensin *in rats* results in a damage of the arterioles, characterized by a deposition of plasma components in the arteriolar walls, followed by an inflammatory cellular reaction (*Olsen* 1970) Evidence of an immunological factor in this reaction was obtained in experiments showing that normal rats which had been pretreated with intravenous injection of thoracic duct lymphocytes from hypertensive rats reacted with a hypersensitivity *of the* delayed type when treated with angiotensin (*Olsen* 1971b)

An inflammatory cellular infiltration *into* hypertensive arterioles or small arteries is not usually described in the human pathology (*Boyd* 1953) or, if described, *it is* considered as inconspicuous (*Pickering* 1968)

The aim of the present work has been to examine whether it is *possible* to recognize an inflammatory cellular infiltration into hypertensively damaged arterioles and small arteries from human subjects suffering from systemic hypertension of different aetiology

MATERIAL AND METHODS

The following groups of hypertensive patients were examined with a view to inflammatory cellular reaction in hypertensively damaged arterial vessels

1 The syndroma of Conn Three patients
2 Pheochromocytoma Four patients
3 Renovascular hypertension Two patients
4 Benign essential hypertension Six patients
5 Malignant essential hypertension One patient
6 Control group Four patients who did not suffer from systemic hypertension or other types of cardiovascular diseases

The kidneys and the periadrenal tissue were fixed in formalin, embedded in paraffin, and cut in five microns thick sections The sections were stained with haematoxylin eosin and finally studied under the light microscope

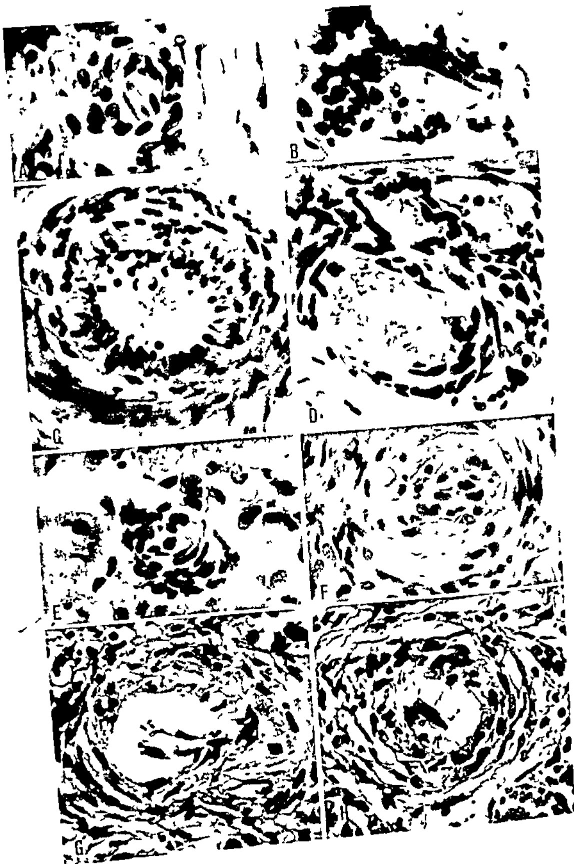
The patients were randomly selected

RESULTS

From every patient, about 20 arterioles or small arteries were examined with view to an inflammatory cellular reaction In about 85

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per cent of the arterial vessels it was possible to recognize such reaction The cellular infiltration was composed of mononuclear cells exclusively which adhered to the surface of the endothelium of the vessels or had penetrated into the tunica media or the adventitia (Fig 1) The inflammatory cellular infiltration was observed in vessels which were damaged by the systemic hypertension, showing either hyaline degeneration or necrosis of the vessel wall But also in vessels in which it was impossible to recognize a hypertensive damage of the wall under the light microscope a mononuclear cellular infiltration was observed The quantity of the mononuclear cells varied to a marked degree from arteriole to arteriole The reaction was less pronounced in vessels which showed hyaline degeneration or necrosis of the total vessel wall and the quantity of the mononuclear cells in the lumen, the tunica media and the adventitia was estimated to be 5 – 8 cells per examined vessel The quantity of the mononuclear cells in arterioles and small arteries with hyaline degeneration or necrosis of only part of the vessel wall was more marked In these cases the number of cells in the lumen the tunica media and the adventitia was counted and found to be 10 – 15 — 35 per examined vessel The same number of cells was found in many arterial vessels in which it was impossible to recognize a hypertensive damage of the wall under the light microscope (Fig 1) Besides the above mentioned difference in quantity of cellular infiltration, the round mononuclear infiltration was found to be more pronounced in the vessels from patients with an acute and severe arterial hypertension than in hypertensive vessels from patients with hypertension of long standing (Fig 1) In the vessels from patients with acute arterial hypertension the degenerative changes were the dominating finding, while the healing processes were sparse Conversely, in cases of mild systemic hypertension persisting throughout many years, healing processes were very often found together with a hyaline degeneration In these vessels an infiltration of round mononuclear cells and spindle shaped fibroblast like cells were observed to be associated with a marked production of connective tissue in the tunica media and especially in the adventitia (Fig 1g and 1h)

Fig 1 demonstrates the inflammatory cellular infiltration into arterioles and small arteries from patients suffering from different types of systematic hypertension

Such inflammatory cellular infiltration was never observed in histological sections from the four patients who did not suffer from systematic hypertension or other types of cardiovascular diseases

Fig 1 The figure shows a mononuclear infiltration into arterioles from patients suffering from a) the syndroma of Conn (duration of the hypertension about 2–3 years), b) pheochromocytoma with a duration about one year, c) and d) renovascular hypertension (duration about one year), e) and f) malignant hypertension (duration about three months) g) and h) benign essential hypertension (duration 5 10 years)

It is possible to observe a mononuclear inflammatory cellular infiltration into the arterial vessels in all the sections These have been cut from the kidneys or the periadrenal tissue The cellular reaction is observed in the lumen the tunica media and the adventitia Besides a round mononuclear cellular infiltration also spindleshaped fibroblasts are seen in figure g) and h) in which a marked production of collagen fibrils have taken place as a sign of a healing process of the damaged arterial vessels Hematoxylin-eosin Magnification 800 ×

DISCUSSION

The present results demonstrate undoubtedly that an inflammatory mononuclear cellular infiltration into arterioles and small arteries takes place in patients with high blood pressure independent of the aetiology of the systemic hypertension, exactly as is the case in rats

In the previously mentioned studies in rats it was made probable that the function of the mononuclear cells is phagocytic and that they further seem to be able to produce collagen connective tissue before and after a transformation into fibrocytes (*Olsen* 1970, 1971a) Furthermore it was found that a hypersensiti

vity of the delayed type was involved in the experimental hypertensive vascular disease (*Olsen* 1971b)

The mononuclear cells found in the arterial vessels in various forms of human hypertension are believed to be a sign of a hypersensitivity of the delayed type and to have the same function as in the experimental hypertension in rats

Other observations support the view that immunological phenomenons are involved in some cases of hypertensive vascular disease *Gardner* et al (1970) found that rats treated with different immunosuppressive methods developed hypertensive vascular disease to a degree less pronounced than that in non treated hypertensive rats *Ebringer* & *Doyle* (1970) observed increased values of immunoglobulins in the serum from patients with severe systemic hypertension and *Paronetto* (1965) demonstrated depositions of complement in arterial vessel walls from patients suffering from malignant hypertension

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The author is very thankful to prof *G Teilum* and prof *E Jensen* The Institute of Pathology University Hospital, Copenhagen who suppl ed the human histological preparations

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Acta path microbiol scand Section A 80, 257–259, 1972

IMMUNOLOGICAL FACTORS AND HIGH BLOOD PRESSURE IN MAN

Systemic Hypertension and Raised Levels of Immunoglobulins in the Serum

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Twelve patients suffering from systemic hypertension without symptoms of collagen disease or other types of systematic disease with the exception of arterial hypertension were examined with a view to concentrations of serum immunoglobulins One patient showed a percent age increment of the immunoglobulins and five patients showed absolute increments of the serum immunoglobulins Two patients showed raised levels of IgA, one patient of IgG, one of IgM and one patient of both IgG IgA and IgM Biopsies were obtained from the kidney, the liver the skeletal muscles and the skin of the latter patient but any signs suggestive of a collagen disease were not found The raised values of the serum immunoglobulins occured in patients in whom the systemic hypertension was of short duration estimated on the basis of the subjective symptoms and the patients were people in whom the arterial hypertension was new-discovered It is discussed whether the raised levels of serum immunoglobulins in hypertensive patients are an index of the hypertensive damage of the blood vessels or whether some cases of systematic hypertension are auto immune diseases

Rats treated with repeated intravenous injections of angiotensin develop an inflammatory cellular infiltration into the hypertensively damaged arterioles and small arteries This cellular reaction follows a deposition of plasma proteins in the walls of the arterial vessels (*Olsen* 1970) Experiments including a transfer of thoracic duct lymphocytes from hypertensive rats to normal recipients showed that a hypersensitivity of the delayed type was involved in the hypertensive vascular disease (*Olsen* 1971)

An inflammatory cellular reaction also takes place in the hypertensive vascular disease in man (*Olsen* 1972) but whether a hypersensitivity of the delayed type is involved in human cases remains obscure It is seldom that an antigen produces only cellular antibodies or only humoral antibodies but a production of both types of antibodies will usually take place when the immunological apparatus is stimulated with an antigen (*Turk* 1967) If an immunological factor were involved in some cases of hypertensive vascular disease in man it might be possible to demonstrate raised levels of serum immunoglobulins

The aim of the present work has been to examine whether some human cases of systemic hypertension show raised levels of serum immunoglobulins

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MATERIAL AND METHODS

Twelve randomly selected patients suffering from systemic hypertension exclusively were examined for immunoglobulins in the serum The concentra *tions of serum IgA, IgG and IgM* were measured using the method of *Laurell* (1966) as modified by *Weeke* (1968)

All the patients were examined with a view to a secondary hypertension This was found in only one patient who developed *systemic hypertension following oral* contraceptives The remaining pa tients suffered from a *primary systemic hyperten* sion One of these was examined with a view to collagen disease and biopsies were obtained from the kidney the liver, the skeletal muscles and the skin

All the examined patients showed normal func tion of the kidneys estimated on the basis of the serum concentration of creatinine

RESULTS

Six of the twelve patients with systemic hy pertension showed raised values of immuno globulins in the serum One of these showed only a percentage increment but not an ab- solute increment of the concentration of the serum immunoglobulins In the remaining five patients with raised levels of serum im munoglobulins, both IgA IgG and IgM were found to be involved in the increment of the serum immunoglobulins Two patients showed raised values of IgA, one patient of IgM and one of IgG One patient showed raised levels of both *IgA IgG and IgM This* patient was examined carefully with a view to a collagen disease and biopsies were ob tained from the kidney the liver, the skeletal muscles and the skin *as mentioned above*

but all these investigations were negative and it was concluded *that the patient suffered* only from systemic hypertension

When the patients in whom values of the serum immunoglobulins were not increased and those in whom levels were raised were *compared in order to detect symptoms* se- condary to the hypertensive *disease and in* dicated by changes in the background of the eyes the electrocardiography findings, X ray of the heart and the concentration *of the serum creatinine, no conclusive* diffe- rence was observed The only characteristic observation was that patients with raised levels of serum immunoglobulins showed a new discovered arterial hypertension of short *duration (maximal one year), estimated on* the basis of the subjective symptoms The age of the patients varied from 21 years to 65 years

Table 1 shows the absolute values of serum IgG, IgA and IgM *in the patients with* raised levels of these immunoglobulins compared with the levels in normal persons

DISCUSSION

Fifty per cent of the examined hypertensive patients showed raised levels of the serum immunoglobulins No symptoms of collagen diseases or other systemic diseases were found with the *exception of* arterial hypertension Thus, it must be concluded that it is possible to demonstrate raised levels of serum immu globulins in some cases of systemic hyperten *sion in human subjects* This indicates an in

TABLE 1 The absolute Levels of IgG, IgA and IgM in the Serum from the Five Hypertensive Patients Who Showed Increased Values of the Serum Immunoglobulins

Patients	IgG	IgA	IgM
B S	9 5 ± 0 4 g/l	0 68 ± 0 04 g/l	1 43 + 0 05 g/l
E M	14 6 ± 0 4 g/l	3 37 ± 0 04 g/l	0 94 + 0 05 g/l
J L	17 6 ± 0 4 g/l	0 93 ± 0 04 g/l	0 72 + 0 05 g/l
R F	12 3 ± 0 4 g/l	3 44 ± 0 04 g/l	1 27 + 0 05 g/l
V V	17 7 ± 0 4 g/l	31 ± 0 04 g/l	1 56 ± 0 05 g/l
Normal	7 2 15 1	0 74-3 06	0 23 1 33

volvement of an immunological factor in some cases of the hypertensive disease The question is How is such immunological involvement brought about? A possible explanation is that the hypertensive degenerative changes of the arterial vessels give rise to a release of antigenic substances from the vessel walls followed by a production of humoral antibodies This explanation presupposes that mononuclear macrophages phagocytize the antigenic substances of the vessel walls From animal experiments we know that mononuclear macrophages penetrate into the hypertensively damaged arterioles and phagocytize products in the walls (*Olsen* 1970) and mononuclear cells have been demonstrated in the hypertensively damaged vessel walls in human subjects (*Olsen* 1972) This means that the requirements for a production of humoral antibodies towards antigens in the arterial walls are fulfilled This explanation is supported by the finding by *Paronetto* (1965) who observed that complement was deposited in the hypertensively damaged arterial walls in patients suffering from malignant hypertension This observation indicates that an antigen antibody reaction possibly had taken place in the arterial walls Furthermore, it has been demonstrated that rats pretreated with different immunosuppressive methods developed hypertensive vascular disease to a degree less pronounced than that of non pretreated rats (*Gardner* et al 1970) *Ebringer* & *Doyle* (1970) found raised levels of serum IgG in patients with severe systemic hypertension and *Köröskenyi* et al (1961) demonstrated a positive complement consumption test in hypertensive patients which was explained as an index for an immunological mechanism in the arterial hypertension

An explanation of the raised levels of serum immunoglobulins in patients with systemic hypertension other than the above mentioned is that some cases of systemic hypertension are auto immune diseases However, this hypothesis has not been supported by observations in the field of human pathology at the present time, but in animal experiments it has been possible to transfer systemic hypertension from hypertensive rats to normal recipients using intravenous injections of lymph node lymphocytes from the hypertensive rats to the normal recipients (*Okuda* & *Grollmann* 1967)

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Acta path microbiol scand Section A 80, 260–262, 1972

PRIMARY SEMINOMA OF THE ANTERIOR MEDIASTINUM

A Case Report with 10-Year Follow-up

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A case of mediastinal seminoma is described A male student, born in 1910, got a cough chest pain, dyspnoea and fever attacks at the end of 1959 One year later, in mediastinoscopy, a tumour 5 cm in diameter was found in the upper anterior mediastinum The histological diagnosis from biopsy was at that time thymic carcinoma, but after re examination seminoma was diagnosed The patient was treated by radiation therapy with a tumour dose of 5400 r Since then he has had no symptoms for 10 years

Mediastinal seminomas are fairly rare, *Molina et al* (1965) report the finding of 35 cases in the literature up to 1965 Since then new cases have been reported in increasing numbers Up to 1969, 63 cases had been described in the literature to our knowledge (*El-Domeiri et al* 1968, *Oancea et al* 1968, *Bagshaw et al* 1969, *Spitzer & Schmitt* 1969)

We found a case of mediastinal seminoma, earlier diagnosed as thymic carcinoma, in a retrospective study of thymomas As it is important for therapy to differentiate this tumour from other mediastinal neoplasms we consider it worth describing

CASE REPORT

A male student born in 1910, previously healthy got pains on the left side of his chest, dyspnoea and fever at the end of 1959 A chest roentgenogram showed a shadow in the hilus region Anti tuberculous chemotherapy was started During 1960 the patient had 3 fever attacks and haemoptysis once The sputum culture for mycobacterium tuberculosis, bronchoscopy and exfoliative cytology from the sputum was negative At the end of 1960 the thorax roentgenogram showed tumour like infiltration in the left hilus region (Figure 1) and the patient was admitted to the University Central Hospital Helsinki, for closer verification of the case

Chest tomography indicated that the tumour was located in the anterior mediastinum in front of the tracheal bifurcation In bronchoscopy the right main bronchus was seen to be somewhat narrowed in an anteroposterior direction In mediastinoscopy a tumour about 5 cm in diameter was found *in the* anterior upper mediastinum It was *firm and* smooth No fluid was obtained by puncture Two *biopsies were taken from the tumour* The histological diagnoses at that time was thymic carcinoma. No tumours were found in the neck, supraclavicular axillary or groin regions The clinical report contained no notes on the testicles but on a check in 1971 nothing special was felt on palpation and the patient had ever seen anything special on them.

Roentgen therapy (230 kV 15 mA) was started through four 10 × 20 cm portals two anteriorly *and 2 posteriorly for 7 weeks with a* 5400 r tumour dose The chest roentgenogram showed a radical recovery after 3 weeks when *a 2100 r* tumour dose had been delivered (Figure 2) the right hilus was normal and on the *left side only a* slight enlargement was seen After the whole roentgen therapy the chest roentgenogram was normal The patient has since then been in good condition

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Fig 1 Chest roentgenogram showing tumour like infiltration in the left hilus region

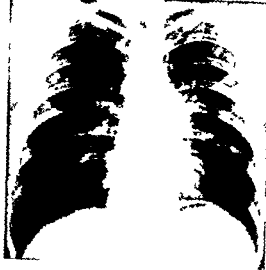


Fig 2 Chest roentgenogram obtained 3 weeks after the beginning of irradiation The mediastinal mass has almost disappeared

Fig 3 Histological section showing typical seminomatous pattern × 170 (H and E)

Histological findings The neoplastic cells were separated in lobules by fine connective tissue septa containing a moderate number of lymphocytes (Figure 3). The tumour cells had distinct cell borders and the cytoplasm was clear and abundant. The centrally located nuclei were large round or oval and the nucleoli prominent The mitotic figures were frequent No Hassall's corpuscles were seen

DISCUSSION

It is probable that seminomas and other germ cell type tumours of mediastinum are primary to that place and not metastases from testicular and ovarian tumours This is supported by the normal palpation finding of the testes in the cases of mediastinal seminoma reported *Kantrowitz* (1934), *Pugsley & Carleton* (1953) and *Fine et al* (1962) have reported four cases in all with teratoid tumours in mediastinum, one with a seminomatous component, whose testes failed to show tumours in serial sections However, it is possible that the primary tumour has regressed spontaneously (*Rather et al* 1954, *Azzopardi et al* 1961) Mediastinum is also a very uncommon place for a metastasis from a testicular seminoma These tumours almost always metastasize to the retroperitoneal lymph nodes first

Lattes (1962) has described four cases of mediastinal seminomas in which Hassall's corpuscles and other thymic tissue were seen At least some of the mediastinal seminomas clearly affect the thymus and possible originate in it The theories of the origin of mediastinal germ cell tumours have been summarized by *Schlumberger* (1946) and *Smith* (1963) The theories mostly referred to are those advanced by *Friedman* (1951), who considers it probable that these tumours originate from displaced germ cells, and by *Schlumberger* (1946), who favours the opinion that they arise in the anlage of the thymus

The only treatment given in the present case was radiotherapy and the patient was symptomless 10 years after the therapy *Bagshaw et al* (1969) have reported a material, partly collected from the literature, comprising 13 patients with mediastinal seminoma treated by irradiation only Nine patients were alive at the end of the follow-up period ranging from one to nine years and the 5-year survival rate was 50 per cent *Bagshaw et al* (1969) recommend radiation therapy in every mediastinal seminoma case, whether or not the tumour is resected

Correct diagnosis of mediatinal seminoma is important because of the radiosensitivity of the tumour Histological diagnosis is not difficult as long as the existence of this tumour in the mediastinum is remembered

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Acta path. microbiol scand Section A 80, 263 266, 1972

ABSENCE OF THE SOLID PART OF THE RIGHT VENTRICULAR MUSCULATURE

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This article deals with the presentation of a 47 year old male patient with total absence of the solid wall of the right ventricle but not of the trabeculae carneae or of the papillary muscles The previous history included attacks of paroxysmal tachycardia and, furthermore angina of effort during the last three years The terminal events were dominated by several types of arrhythmias The autopic findings revealed no degenerative or inflammatory changes of the myocardium The coronary arteries were normal except for non-contributory minimal atheromatous deposits Absence of the solid part of the right ventricular musculature probably represents a congenital defect

Only 10 cases of partial or complete absence of the solid part of the right ventricular myocardium have been reported (1, 2 – 12, 14) Some of these cases were combined either with similar changes in the other heart chambers, predominantly in the right atrium (7, 8, 10, 11) or with congenital disorders of the heart (12) The theory has been put forward that this lesion probably represents a primary developmental defect of the heart (14) By exclusion, this also seems to be the reasonable pathogenic explanation in the case described below

CASE-REPORT

According to reports from his ordinary family – doctor in England, this 47 year-old male English man had been suffering from attacks of paroxysmal tachycardia since adolescence These attacks were easily treated by procainamide i m In 1965 he began to feel *mild angina* of effort. He was treated in a hospital in England in November, 1967 An electrocardiogram revealed wide spread T wave changes "indicating ischaemic heart disease" After his discharge from the hospital he complained of increasing dyspnoea Otherwise, he was free of symptoms until July 1968 when an episode of tachycardia and diffuse thoracic pains suddenly occurred He was then admitted to the medical out patient department of Borås Hospital His condition appeared to be good He showed no signs of cardiac decompensation Physical examination of the heart disclosed decreased intensity of both sounds The blood pressure was 130 systolic, 90 diastolic An electrocardiogram demonstrated a Q_3 T_2-pattern and coronary T-waves in V_1-V_4 and, moreover episodic bouts of ventricular tachycardia After injection of 0,2 gm procainamide i m a sinus rhythm was obtained About one hour later ventricular ectopics appeared and he was then transferred to the medical ward

Laboratory tests The values of haemoglobin, white-cell count, serum creatinine, various liver function tests and sedimentation rate were all with in normal values

X ray films of the heart were taken with the patient in a recumbent position Consequently a closer analysis could not be performed The heart was somewhat enlarged and the pulmonary outflow tract seemed to be dilated There was no pulmonary vascular congestion

During the following weeks several types of arrhythmias appeared, e g supraventricular and multifocal ventricular ectopic beats, as well as bouts of nodal tachycardia Conventional anti arrhythmic drugs were of only transient benefit A pacemaker

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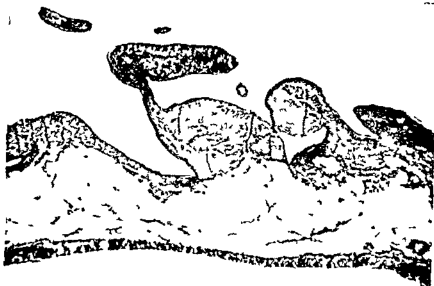


Fig 1 Section from the wall of the right ventricle The solid part of the myocardium is absent and there are only papillary muscles and some thin trabeculae carneae Between the endocardium and the epicardium a mature adipose tissue Weigherts method for elastic fibres × 10

was therefore inserted with the negative electrode transvenously attached to the right ventricle The excitability threshold value was 1,25 volt However various kinds of rhythm disturbances were recorded on the following days Ventricular fibrillation occurred on the twenty second day in hospital Attempts of resucitation were of no help and the patient died shortly after

Autopsy The heart weighed 450 gm The superior and inferior venae cavae drained into the right atrium which was greatly dilated and moderatly hypertrophied Foramen ovale was closed The tricuspidal ring was normal The right ventricle was markedly dilated and the wall was very thin mostly 1 2 mm thick and there were large areas where no muscle fibres were visible The thinning involved the parietal wall which was overlaid by few and thin trabeculae carneae The pulmonary orifice was normal

The electrode of the pacemaker was situated in the right ventricle but not fixed to the wall The wall of the left ventricle was slightly hypertrofied The myocardium showed no evidence of fibrosis or necrosis The aortic and left atrioventricular orifices appeared to be normal The coronary arteries arose in their normal location and there was equal distribution of the right and left coronary arteries between the chambers of the heart Minimal segmental atheromatous deposits were found in the anterior descending artery but there was no evidence of coronary occlusion and no appreciable reduction in the size of the coronary lumens

The lungs were dark and anthracotic and moderately oedematous

The liver and the spleen were congested

The rest of the autopsy did not reveal any significant pathological changes

On microscopic examination the myocardial bundles of the left ventricle showed evidence of slight hypertrophy No cicatrization or myocardial necrosis was found Section through the right ventricular wall revealed a normal or slightly thickened endocardium composed of interlacing bundles of fibrocollagenous tissue lined by endothelial cells The solid part of the myocardium was replaced by mature adipose tissue containing medium sized muscular arteries and rare minute nests and bundles of myocardial cells In the subendocardial part of the wall there were few and thin trabeculae carneae or papillary muscles (Fig 1) The coronary arteries were widely patent

DISCUSSION

Osler (7) described a remarkable heart of an adult showing an extreme degree of dilatation of all the chambers without the hypertrophy that usually occurs The parchmentlike thinness of the mostly transparent walls was confirmed by re-examination of this specimen (10) and the term "parchment heart was introduced The possible existence of trabecu

lae carneae was not stated but the papillary muscles were thin, and, at least partly, the ventricular walls consisted only of endocardium Since then a small number of partial or total absence of the right ventricular musculature have been reported, including only six infants (1, 2, 4, 6, 8, 12, 14) and three adults (5, 9, 11) In two of the cases published, the lesion was extended to the right atrium (8, 11) In one patient this anomaly was combined with an Ebstein's malformation (12), and in another an interstitial myocarditis of the left ventricle was also found (8, 12)

The course of disease was fairly similar in the six infants reported The clinical picture was characterized by a severe congestive heart failure, a highly dilated right ventricle and an almost total absence of the myocardium of the right ventricle Thus in the first reported case (14) no myocardium was visible except at the base of the chamber, the pulmonary conus and a small portion of the posterior aspect of the wall

The first adult case was reported in 1952 (11) On admission to the hospital, this 24-year old woman had throughout eleven months a history of swelling of her legs, variable fever, dyspnoea and cough As in our case, periods of arrhythmia were observed She died 2½ years after the onset of the symptoms of what appeared to have been congestive heart failure The diagnosis became clear at autopsy

The second adult case was a 47 year old woman who died of a subarachnoid haemorrhage (9) This case differs from ours by the presence of cyanosis and polycythaemia, right atrial hypertrophy and an intraventricular conduction disturbance on electrocardiogram The autopsy revealed that large areas of the wall of the right ventricle were devoid of muscle fibres

The third adult case showing this abnormality was a 57 year-old man who died of a myelogenous leukaemia (5) This patient differed from the present case in that he always had felt himself healthy and, furthermore, in that the electrocardiograms showed only small and non specific changes On the other hand, there are certain similarities between this case and that described in the present paper Thus, the autopsy revealed an extremely thin wall of the right ventricle with preserved trabeculae carneae and papillary muscles On microscopic examination, a discontinuation of the myocardial bundles in the right ventricle was found, but there was no fibrosis or necrosis The other heart chambers showed no alterations

In all cases previously described, as well as in the case under discussion, there were no or minimal deposits in both coronary arteries Therefore a healed myocardial infarction seems unlikely as the cause of this abnormality Because of the microscopic picture, a destruction of the myocardium by an inflammatory or degenerative process likewise seems improbable The cause of this selective heart muscle lesion thus remains unknown, but may represent a true primary congenital defect in the development of the heart (14) and may be explained by *Davis'* (3) observations of the early stages of the embryology of the human heart At the 4 somite stage the myocardium of the further heart consists of a bilaterally symmetrical mantle of mesenchymal cells enclosing an endothelial plexus The mantle comprises a pair of cardiogenic folds, one of which forms the epicardium and myocardium of the right ventricle and the other those of the left ventricle By the 9 somite stage complete fusion of the paired cardiogenic folds has occured According to *Uhl* (14), an injury to the right cardiogenic fold before the fusion may result in the development of a heart without myocardium in the right ventricle However, Uhl s explanation is contradicted by the observations of *de Vries* & *Saunders* (16), according to whom the paired cardiogenic folds are fused to a straight bulboventricular tube which will be differentiated in three parts, one cranial (the aortic sac) one caudal (the early embryonic ventricle, later the left ventricle) and one rather small midportion (the bulbus cordis, later the right ventricle) Therefore the right ventricle is developed from the left as well as from the right cardiogenic fold How

ever, it appears likely that the distal part of the cardiogenic fold contributes more to the right trabeculated ventricle than the distal part of the left cardiogenic fold Thus, injury to the right cardiogenic fold might result in the development of a heart without myocardium in the right ventricle Supposing that these embryologic considerations are relevant in the cases described it seems unlikely that the lesion always should be confined to the right ventricle Thus, an involvment of the right atrium was also present in two cases (8, 11) and in *Osler's* original case, all four chambers were affected (7) *Tenckhoff et al* (13) discussed a 16 year old boy in whom autopsy disclosed a paper thin right atrial wall and the authors arrived at the conclusion *that this represents a form of the same ano*maly

A high degree of reduction of the myocardium of the right ventricle obviously results in a greater incapacity to cope with the venous flow, ending up in severe decompensation and a high risk of early death In the three adult cases previously described and also in the present case the trabeculae carneae and papillary muscles remained wholly or partially while the solid part of the myocardium was almost totally absent and replaced by mature adipose tissue

These quantitative differences may explain the variation in the course of disease and in the age of the patients reported It seems evident that the right ventricle was contributing little to the circulation However, haemodynamic observations in a case with degenerative changes in the walls of the right and left ventricles (15) strongly favours the assumption that the right atrium in cases with a defect capacity of the right ventricle is functioning as a pumping chamber for propelling blood into the pulmonary circuit

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Acta path microbiol scand Section A 80, 267–283, 1972

THE ULTRASTRUCTURE OF HUMAN AND MURINE ASTROCYTES AND OF HUMAN FIBROBLASTS IN CULTURE

Elizabeth H Macintyre Jan Pontén and A E Vatter

A study was undertaken to seek fine structural differences between normal and maglinant astrocytes and normal fibroblasts in cell culture A series of human adult normal astrocytes and malignant astrocytes (including RSV transformed cells) whose cultural characteristics had previously been reported formed the basis of the study Also included were matched normal human fetal fibroblasts and astrocytes (from the same fetus) and methylcholanthrene induced murine malignant astrocytes Pellets of cells scraped from their culture vessel and sectioned as well as *in situ* preparations were examined The fibroblasts were the only cells whose external microfibrils were collagenase sensitive and considered to be tropocollagen. Both the benign and the malignant astrocytes had intracytoplasmic microfibril bands (gliofibrils) and some had external collagenase resistant microfibrils Malignant astrocytes had a much more developed membrane system (used in its widest sense) than normal astrocytes, had many free ribosomes and some had morphologically abnormal mitochondria. Virus was seen only in the murine malignant astrocytes was abundant and of the murine leukemia virus group Areas interpreted as representing fusion in progress were seen at the surface of some RSV transfomed giant tumor astrocytes The fine structure of one cell type from an IgG producing culture from human glioblastoma corresponded with that reported for immunocytes

The cytology and growth kinetics of human astrocytes in cell culture have been described (*Ponten & Macintyre* 1968) This series was obtained by culturing routinely all neurosurgical specimens taken over a prolonged period These biopsies included tissues of tumor and non- tumor origin from brain Variants trans formed by Rous sarcoma virus (RSV) were derived from these cells (*Ponten & Macintyre 1968, Macintyre et al* 1969) The aim of the present study has been to examine the fine structure of these same cultures, in order to define criteria for their identification, as already established for astrocytes in whole brain (*Luse* 1958, 1960, 1968, *Maxwell & Kruger* 1965, *Eager & Eager* 1966) The work has been extended to include murine tumor astrocytes, induced by a chemical carcinogen (*Zimmerman & Arnold* 1941), as well as human fetal astrocytes and matching fibroblasts

Received 9 ix.71

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MATERIALS AND METHODS

The studies concentrated on human cells Culture methods and conditions of cell growth were as already described (*Pontén & Macintyre* 1968, *Macintyre et al* 1969) Human fetal cells were grown from two and three month old therapeutic abortions – astrocytes and fibroblasts being obtained from cerebral and pulmonary tissues respectively Five fetuses were cultured, and since the fine struct

ure of all was comparable for the two cell types, the matched cells from a three month old fetus – HFNG5 (astrocytes) and HFF5 (fibroblasts) were chosen. The other human cells studied came from the series of routine neurosurgical tissue cultures previously reported (*Pontén & Macintyre* 1968, *Macintyre et al* 1969). 125CG and 158CG were normal human adult astrocyte cultures while 87MG, 105MG, 118MG, 120MG, 138MG were the human tumor astrocyte sources. From these cells 125CG, 105MG and 118MG were chosen as typical representatives of the normal and tumor astrocytes respectively. A separate section is devoted to 119MG because of the associated immunocyte production.

Murine tumor astrocytes (MMG) from astrocytomas were grown by described methods (*Pontén and Macintyre* 1968) using both grid and direct culture procedures. The original tumor had been induced *in vivo* by intracerebral implantation of methylcholanthrene in C3H mice (*Zimmerman & Arnold* 1941, *Kirsch* 1963), and carried by successive subcutaneous implants in the same inbred strain. We are indebted to Dr. *Wolff Kirsch* for supplying the tumors for culture. A histologically identical tumor was also produced by injecting the cultured MMG cells into mice. In one such experiments, 7×10^6 MMG1 cells at transfer 58 in culture were injected into each of three female C3H mice, one month old. Three weeks later all three had a tumor at the injection site, the largest measuring $3 \times 1 \times 1$ cm.

Morphological Studies by Electron Microscopy. Cells were fixed in a mixture of glutaraldehyde and osmium tetroxide (*Hirsch & Fedorko* 1968). The stock solutions of 2.5 % glutaraldehyde in 0.1 M cacodylate pH 7.4 and of 1 % osmium tetroxide in 0.1 M cacodylate pH 7.4 were maintained separately at 4°C. To prevent precipitation of osmium by the glutaraldehyde, these solutions were brought to 0°C in an icebath and mixed (one part glutaraldehyde plus two parts osmium tetroxide) immediately prior to use. The mixture remained clear, colorless and no precipitate formed. 0.5 ml of this mixture was added directly to the culture medium (5 ml) without disturbing the cells and immediately after removing the plates from the 37° incubator. The liquid was drained within a minute and replaced with the 0°C mixture of glutaraldehyde and osmium. The preparation was placed on ice and after five to ten minutes the cells were removed by scraping and pelleted by brief (one to two minutes) centrifugation in the cold. The mixture for fixation was replaced, the total fixation time was thirty minutes.

The above technique was modified for preparation of RSV transformed human tumor astrocytes (EH 118MG cells), which produce both surface attached and free cells (*Macintyre et al* 1969). After the initial one minute fixation the attached cells were scraped off into the medium which already had the free cells and the cell fixative mixture was pelleted by brief centrifugation before the liquid was removed. By this means the free cells were not discarded. The rest of the fixation procedure was performed as above. When segregation of the free and attached EH 118MG cells was required, the medium containing the free cells was pipetted into a centrifuge tube and processed as already outlined in this paragraph for the mixture of free and attached cells. The attached EH 118 MG cells, on the other hand, were washed twice with complete medium at 37°C to remove any free cells before fixation and processing.

After the completion of the fixation period the pellet was rinsed several times with 0.1 M cacodylate buffer pH 7.4 at 0°C. This was followed immediately by two rinses with cold 0.1 M acetate buffer at pH 6.3 containing 0.25 % uranyl acetate (*Hirsch & Fedorko* 1968). The pellet was left in contact with the uranyl acetate solution for twenty minutes at 0°C. It was then washed with several changes of cacodylate buffer and dehydration of the pellet followed through 70, 80 and 97 % hydroxypropylmethacrylate in water, allowing five minutes for each concentration. The cells were further infiltrated with a series of hydroxypropylmethacrylate and Epon mixtures and embedded in Epon. Sections of various preparations were stained with lead citrate (*Reynolds* 1963) to increase contrast.

In cases where cell surfaces were to be examined the cells were prepared *in situ* in the culture vessel. This was made technically feasible by growing cells on a plastic layer. Prior to the inoculation of cells 3 to 5 ml of Epon were introduced in the culture vessel and polymerized at 50–60°C as a layer covering the bottom of the vessel. Cells were seeded and cultured as above. Cultures were fixed by the addition of 0.5 ml of fixative to the culture bottle and the medium fixative mixture removed after one to two minutes. 3.5 ml of fresh, cold fixative was added to the bottle which was then placed in an icebath for thirty minutes. All subsequent procedures were identical to those described above except that the cells remained *in situ*. Dehydration and embedding were carried out in the vessel. After polymerization of the Epon, the plastic sandwich containing the cells was removed by breaking the container.

Cytochemical Studies. Cell cultures were investigated to determine whether or not the extracellular fibrillar mats in high density cultures were collagenase sensitive. Unfixed cultures were incubated for 1 hour at 37°C in 5 ml of buffer adjusted to pH 7.4 that contained 0.137 M NaCl – 0.0054 M KCl – 0.003 M $CaCl_2$ – 0.005 M $MgSO_4$ – 0.0025 M Tris and 0.4 mg collagenase (2.36 units/mg, Worthington, Freehold, N. J.) and

then fixed with the mixture of glutaraldehyde and osmium and processed *in situ* In preparations where mucopolysaccharide detection was desired specimens were treated according to *Luft* (1964) with ruthenium red which shows a particular affinity for highly polymerized acid polysaccharides The specificity of the reaction was established according to the methods of 1 *Revel* (1964) using colloidal thorium on unembedded cells and thin sections with and without prior treatment by hyaluronidase (Worthington Freehold N J) and 2 *Neutra* & *Leblond* (1966) using the peracetic acid and beta glucuronidase (Worthington Freehold N J) sequence

Cells whose cytoplasm was PAS positive (Armed Forces Institute of Pathology Manual 1960) were investigated for glycogen content The enzymatic digestion technique of *Rosa* & *Johnson* (1967) was used with *α* amylase as the glycogenolytic agent The presence of cellular acid phosphatase was sought first by the classical histologic Gomori technique

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the presence of acid phosphatase in single membraned intracytoplasmic structures identified as lysosomes Lipid inclusions were identified by their positive staining with oil red O in ultrathin sections (*Tzitsikas et al* 1962) The latter technique was developed originally for use with material embedded in polyester resin It is also successful with Epon embedded material (*A E Vatter* unpublished observation)

Other techniques Stereomicrographs of sections of *in situ* preparations were studied from regions where cell fusion appeared to be a possibility (*Macintyre et al* 1969) using a stage that was tilted from 8° left to 8° right

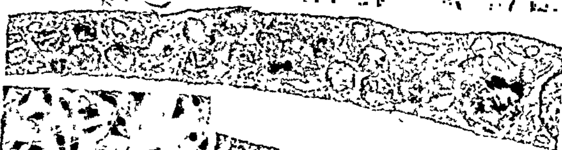
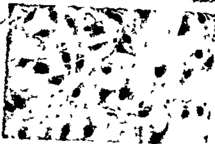
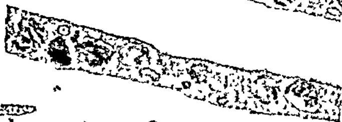
Phagocytic activity was shown by adding 0 05 ml of a fine colloidal suspension of a non toxic carbon (Guenther Wagner Pelikan Werke Hanover Germany Batch # C11 1431A) per 5 ml culture medium (Macintyre et al 1969) The free extracellular carbon was rised off after two hours incubation and the cells fixed either for electron microscopy as already detailed or for histology (100 % ethyl alcohol) The number of cells carrying intracellular carbon was counted under the light microscope This gave an estimate of cellular *phagocytic activity* The intracellular localization of the carbon was shown by electron microscopic study (*Macintyre et al* 1969)

RESULTS

Morphology of the Cell Types

The compilation of electron microscopic findings will be preceded by a brief review of the main characteristics of the different cell types HFNG5 astrocytes grew as a loose monolayered network with ill defined individual cytoplasmic borders (Fig 1, insert) to attain a terminal density of $1\,0 \times 10^5/cm^2$ HFF5 fibroblasts grew in whorls to a terminal density of $2\,2 \times 10^5/cm^2$ (Fig 2, insert) The astrocytes grew much more slowly than the fibroblasts, and their cytoplasm was more diffusely spread out At terminal cell density, the cytoplasmic borders of neighbouring astrocytes usually were separated from one another This separation was exaggerated by the fixative for light microscopy (100 % ethyl alcohol) (Fig 1 insert) Normal human astrocytes (*Fig 3, insert*) *represented by* *125CG*, attained a terminal cell density of $0\,5 \times 10\ /cm^2$, proliferating even more slowly than HFNG5 They showed the strictest contact inhibition of all cells described here (*Pontén et al 1969*), and grew as a pavement of cells with extremely tenuos cytoplasm 158CG was comparable Human tumor astrocyte lines (87MG, 105MG, 118MG, and 138MG) attained a terminal density of $1\,3 - 1\,6 \times 10^5/cm^2$ as mitosing multilayers of spindle cells (Fig 4) or more rarely, closely packed epithelioid cells (Fig 5) 105MG and 118MG were chosen to represent the characteristic fine structural features of the tumor astrocyte group 119MG cells (*Ponten* & *Macintyre* 1968) encompassed two distinct cell types tumor astrocytes (Figs 9 10, single arrows) and immunoglobulin producing cells (Figs 9, 10 double arrows) The former cells died out in culture after several transfers The immunocytes did not Samples were studied while the tumor astrocytes were growing well RSV transformed tumor astrocytes (EH 118 MG) (Fig 14) cycled between attached and free growth states and continued proliferating as long as nutrient was available (*Macintyre et al* 1969) The number of giant cells increased with the length of time the cells grew undisturbed probably because of the fusing capability of RSV (*Moses and Kohn* 1963)

With regard to murine cells in culture (Fig 19 insert B) the histology of the ori-


ginal primary high grade astrocytoma was still retained in the grid culture of this astrocytoma (Fig 19, insert A), which was sacrificed after two months in vitro (Similar observations have been made for grids of human astrocytes (*Ponten & Macintyre* 1968)) The same histologic pattern was seen in tumors caused by injecting the cultured cells into mice The murine tumor astrocytes were shown to harbor murine leukemia virus in large quantities The presence of virus like particles in the cultured cells was first noted under the electron microscope (Figs 19, 21, 22, 23) Subsequently, Dr *Lloyd J Old*, Sloan Kettering Institute for Cancer Research New York, was kind enough to test the MMG cells and found by immunodiffusion methods that the MMG cells were strongly positive for murine leukemia virus group specific (MuLV sl) antigen (*Geering et al* 1966, *Nowinsky et al* 1968) They did not contain murine mammary tumor virus group specific antigen (MTV sl) (*Nowinsky et al* 1968)

All the normal astrocytes and fibroblasts had a finite *in vitro* life span, and were examined in phase II of growth (*Hayflick & Moorhead* 1961) The tumor astrocytes in this study had an infinite life span in culture, with the exception of 120MG, and were studied after varying numbers of transfers and therefore of generations *in vitro* for each line The fine structure of individual cultures was viewed both when the cell number was sparse and at terminal cell density The murine tumor astrocytes were discarded after demonstration of their MuLV sl content and so their life span *in vitro* is not known

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Fig 1 and insert *Human fetal astrocytes* (HFNG5) These normal cells grow in vitro as a monolayer which attains only a low terminal cell density (insert May Grunwald Giemsa Magn. × 100) Their cytoplasm contains a smaller number of organelles than the other cells investigated in this series (Magn × 3 600)

I , *(HFF5)* in the e they maintain monolayer status er terminal density than their matched astrocytes (insert May-Grunwald-Giemsa Magn × 100) The cytoplasm contains a greater number of organelles than in the HFNG cells above The oblique cut of the cell surface accentuates the microfibrillar mat Lipid inclusions (arrow) were present. (Magn × 5 000)

Fig 3 and insert. *Human adult astrocytes* (125GG) These normal cells attain the lowest terminal cell density of all the cultures investigated in this work They maintain very strict contact inhibition and have a widely spread thin cytoplasm (insert May Grunwald-Giemsa Magn × 100) The unique ultrastructural feature of these cells was their accumulation of large numbers of lysosomes (Magn × 14 000)

Cellular Fine Structure

1 *Normal human fetal astrocytes (HFNG 5) (Fig 1)* The most interesting feature of the HFNG cells was found after cell to cell contact was established This was the appearance of a fine microfibrillar mat between contiguous cells The microfibrillary material was not banded, was approximately 50 Å in diameter, and resistant to collagenase The cytoplasmic surface was indistinct, so the exact border could not be defined A thin surface layer of acid mucopolysaccharide was present Microtubules of approximately 120 Å in diameter, coursed through the cytoplasm at intervals, and others arranged in bundles were oriented parallel to the long axis of the cell. In actively proliferating cells no external fibrils were seen and the cytoplasmic membrane was intact At areas in such cells however small groups of similar microfibrils lay immediately under the cytoplasmic membrane and were oriented in parallel with it or coursed through the cytoplasm Their chemical nature is unknown The single nucleus was usually ovoid The nuclear chromatin was finely granular the nucleoli were very large Occasionally the cells contained lipid inclusions and glycogen The cells phagocytosed carbon poorly

2 *Normal human fetal fibroblasts (HFF5) (Fig 2)* The fibroblasts were more compact cells, and were shorter than their companion astrocytes Their cytoplasm was packed with organelles-nests of mitochondria and an extensive network of granular endoplasmic re-

ticulum, whose cisterns were usually filled with products of undetermined nature Membrane bound ribosomes predominated over free polyribosomes Glycogen granules were absent The Golgi system was easily found, but not hyperplastic Lysosomes were small and unobtrusive Lipid inclusions (Fig 2, arrow) were often prominent, especially adjacent to nuclear poles

Nuclear chromatin was more coarsely granular than in the corresponding astrocytes nucleoli were large The free surface of the fibroblasts often appeared indistinct, even in proliferating states, because the irregular contour led to many oblique cuts In high density cultures, a thick mat of coarse, irregular fibrils, 50 Å to 70 Å in diameter (Fig 2) was associated with the surface, providing a matrix between adjacent cells The fibrils were much more abundant than those of astrocytes and they sometimes were beaded, but they were not banded They were digested when treated with a collagenase preparation The fibrils were interpreted as being tropocollagen The cells phagocytosed carbon well Approximately 50 % of the cells had phagocytosed carbon after the usual two hours' exposure period (*Macintyre et al* 1969) The external surface of the cells was covered by a thin layer of mucopolysaccharide material

3 *Normal human adult astrocytes (125 CG)* *(Fig 3)* These were very long and slender cells, whose surface appeared devoid of fibrils The disposition of organelles resembled that of the fetal astrocytes External microfibril accumulations were less frequent in regions where cell to cell contact was observed Lipid-like accumulations and glycogen granules were not detected in these cells The cytoplasm contained abundant lysosomes (*Macintyre et al* 1969) These were universally present in the tips of the adult astrocytes, and often were so numerous that they filled the cytoplasm from the tips to the nuclear poles The lysosomes varied in size from one to four microns in diameter, and at times the cytoplasm was quite distended by these organelles

4 *Human tumor astrocytes* (105MG 118 MG) (Figs 6, 7 and 8) Extensive development of many types of membrane (*Wallach* 1968) and marked variability in kind and number of organelles characterized the tumor astrocytes (*Macintyre et al* 1969) This applied in different parts of the same cell to different cells from the same culture, and to different tumor lines It also held true for these cells after few or many generations in culture, and at high and low cell density

Perhaps the most remarkable of all these features involved the Golgi system (Figs 6, 8) This was hyperplastic and extended considerable distances from the paranuclear area Its cisterns were in a variable state of dilation The rest of the protein producing apparatus as represented by ribosomes free of membranes (polyribosomes) or membrane-bound (granular endoplasmic reticulum), was likewise abundantly represented The ribosome excess is demonstrated on light microscopy by marked cytoplasmic basophilia Glycogen granules were present in many cells (Fig 6) (*Maxwell & Kruger* 1965)

There was an intricate microtubular system, which ran in single strands or small, parallel bundles throughout the cytoplasm, each microtubule being approximately 120 Å in diameter At the cell border there was sometimes no special feature, at other times, numerous microtubules lay subjacent and pa-

Figs 4–8 Human tumor astrocytes (118MG & 105 MG) In culture, there are two main morphological cell types – the spindle (Fig 4) and the epithelioid (Fig 5), Both attain a much higher terminal cell density than the normal astrocytes and multilayering is found (Fig 4, May Grunwald Giemsa Magn × 200 Fig 5 unstained live culture, oblique lighting, Magn × 200) The examples shown in this instance 1181N1 (spindle) and 118D1 (epithelioid), were isolated from the same parent line 118MG The cytoplasm of all human tumor astrocytes showed a marked variability in organelle number and type Some of the more remarkable features were the glycogen granules (105MG, Fig 6, arrow) the extensive development of microtubules and microfibrils (1181N1 Fig 7) and the hyperplastic Golgi complex (118 MG Fig 8) (Fig 6, Magn × 27 000 Fig 7 Magn × 20 000, Fig 8, Magn × 90 000)

rallel to the cell surface (Fig 7) and communicated freely with the random tubules of the cytoplasm The cytoplasmic border was sometimes well delineated more often indistinct, again the result of oblique cuts Microfibrils were present in the peripheral portion of the cytoplasm especially in association with the peripheral collections of microtubules Externally, *microfibrils were present* analogous to those found in association with the astrocytes These showed no periodicity Again, as with the microfibrils of the fetal astrocytes their chemical nature is unknown Collagenase did not digest them

A surface layer of acid mucopolysaccharide was present on two morphologic types of 118MG 1181N1 (spindle) and 118D1 (epithelioid) This was much more prominent than that seen in the fetal cells The other tumor astrocytes were not tested for this Lysosomes of all stages were common (Figs 6 and 8) some were distended to dimensions comparable with those already described for adult astrocytes However, whereas the adult cells were in contact when lysosomal accumulation occurred in tumor cells this distension and accumulation of lysosomes was found at all growth stages, and in various areas of the tumor cell cytoplasm

Mitochondrial nests were frequent, and although the majority of these organelles was normal in morphology, definitely abnormal forms were found in the tumor cells – a feature not shared with normal cells Almost all the cells phagocytosed carbon well evidence of pinocytosis was rare No lipid or virus like inclusions were found in association with these cells Nuclear chromatin was often clumped, but nuclei and nucleoli were not remarkable except for some pleomorphism Microtubular formation and *external microfibril* accumulation seemed if anything more prominent in the epithelioid variety of tumor *astrocytes* (Fig 5), otherwise there was no *structural difference* between them and the spindle shaped sarcoma morphological type (Fig 4)

5 *Human tumor astrocytes and immunoglobulin-producing cells* (119MG) (Figs 9, 11, 12, 13) The two morphologically distinct cell types apparent in culture (Fig 10) – the large, irregular tumor astrocyte and the small round immunocyte were distinguishable with ease in the electron microscope (Fig 9) The fine structure of these cells was examined at an early passage, before the tumor cells disappeared The tumor astrocytes differed in two main ways from those described above (paragraph 4) First, they had numerous slender, anastomotic projections from the free surface (Fig 9) Contiguous tumor cells came together in an interlacing mosaic without fusion (Fig 9) There were few intervening microfibrils Second 119MG culture were unique in having dead cells, the recognizable ones of which appeared to be tumor cells These and cell debris were phagocytosed by the tumor cells Otherwise, ultrastructural features resembled those described above for the other human tumor astrocytes with a greater degree of nuclear pleomorphism a greater number of abnormal mitochondria and fewer lysosomes

The immunocyte volume was taken up mainly by the cell nucleus (Fig 9) The pe-

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Figs 9–13 Human tumor astrocytes and immunocytes (119MG) The tumor astrocytes of 119MG were present *in vitro* as a large cell with extensive, voluminous cytoplasm and an immense eccentric rounded nucleus which is about three times the size of the immunocyte nucleus and contains a large nucleolus (Fig 10 single arrow) The immunocytes (Fig 10, double arrow) are more numerous in the culture have a round eccentric nucleus approximately 10 microns in diameter bound by a narrow rim of *highly basophilic* cytoplasm (Fig 10 May Grunwald Giemsa Magn × 400) within which dilated granular endoplasmic reticulum cisterns (Fig 11 arrow) lie parallel to the plasmalemmal surface The two cell types are easily distinguished in electron microscopy The tumor cells (Fig 9, *single arrow*) have numerous filiform protrusions There are many areas of intimate contact between the two cell types (Figs 9 12 13) with apparent membrane fusion However stereomicroscopic examination of these areas shows that each cytoplasmic membrane is intact and that there is no membrane fusion (Fig 9 Magn × 8 000 Fig 11, Magn × 36 000 Fig 12 Magn. × 45 000 Fig 13 Magn × 50 000)


13

ripheral rim of cytoplasm carried few membranes, no abnormal mitochondria, and many polyribosomes There were occasional cisterns of the granular endoplasmic reticulum, which were usually parallel to the cytoplasmic surface The cisterns of these figures were slightly dilated and contained amorphous material (Fig 11) The cytoplasmic membrane was intact and the surface was smooth The area of contact between tumor cells and immunocytes was scrutinized closely in many different areas There were several instances, typified by the examples shown (Figs 12, 13), where the cytoplasmic borders of the two cell types were indistinct, but in none of the many sites examined was there evidence of organelle crossover On examination of stereomicrographs, cytoplasmic membranes were intact between such closely aligned cells These areas were considered therefore not to represent cytoplasmic fusion Neither virus nor lipid like particles were seen

b RSV transformed human tumor astrocytes (EH 118MG) (Figs 15–18) Further study of the RSV transformed astrocytes confirmed the fine structural details which have already been described (*Macintyre et al 1969*) Briefly, they differed from the other human tumor astrocytes in having fewer lysosomes, a less well developed Golgi and granular endoplasmic reticulum system and showing in some cases blunt cytoplasmic protrusions (Fig 16) Abnormally shaped mitochondria were especially prominent Microtubules were present in the stellate, round and giant cells, but were not a prominent feature as in the tumor astrocytes already described

The arrangement of the microfibrils differed among the three cell types In all these cells microfibrils were found throughout the cytoplasm in apparently random patterns In the case of the stellate cell (Fig 15), there was a remarkable arrangement of these microfibrils into parallel arrays 0 2—1 micron in diameter (Fig 15) These bundles sometimes divided and joined other bundles forming an anastomosing complex through the cytoplasm This pattern was not seen in the round cells This difference is all the more remarkable considering that the Rous cells cycle between the stellate and round forms Microfibrillar arrangement within the cytoplasm of the giant cells varied from a widely distributed meshwork to parallel arrays in bands as described in the stellate cells Under parts of the plasmalemma in some giant cells there was evidence of a fine meshwork which extended about 0 5 micron inward into the cytoplasm The giant cell was thus a larger scale version of the other Rous transformed tumor cells

Since cytoplasmic and nuclear fusion had been a characteristic feature of the transformed cells (*Macintyre et al 1969*), a study was

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Figs 14–18 RSV transformed human tumor astrocytes (EH 118MG) In established cultures (Fig 14) the Rous transformed cells show three morphological types – giant stellate (single arrow) and round (double arrow) (Fig 14 Magn × 200) The round cells grow in multilayered heaps from which viable cells shed These free round cells assume stellate shape when seeded to a fresh culture surface The cytoplasm of the stellate cells (Fig 15) is traversed by broad anastomosing bands of microfibrils The plasmalemmal surfaces of adjacent cells are closely apposed and interdigitate but no fusion is present Free round cells (Fig 16) often have many broad based cytoplasmic projections The external acid mucopolysaccharide layer is represented by a dark irregular precipitate which serves in this instance to illustrate that contiguous cytoplasmic surfaces have not fused

The tumor giant cells in less dense areas of cell growth have sometimes long narrow necked pseudopodial extensions which interdigitate with similar projections from contiguous cells (Figs 17, 18) In many instances there is no fusion despite intimate contact between these extensions from adjacent cells In other areas of contact the intact cytoplasmic membranes appear replaced by a line of microvesicles (Fig 17, single arrow) sometimes underlaid by microfibrils (Fig 17 double arrow) Identical microfibrillar arrangements are seen under adjacent intact plasmalemma (Fig 17, triple arrow) Microvesicles aligned in the above way are considered to represent a site of cytoplasmic fusion in progress at the time of fixation The difference in the cytoplasmic density of contiguous cells at a site where fusion appears to have started is shown in Fig 18 Stereomicroscopy of this area shows no intact cytoplasmic membranes (Fig 15 Magn × 16 000 Fig 16 Magn × 9 500 Fig 17 Magn × 13 000 Fig 18 Magn × 34 000)



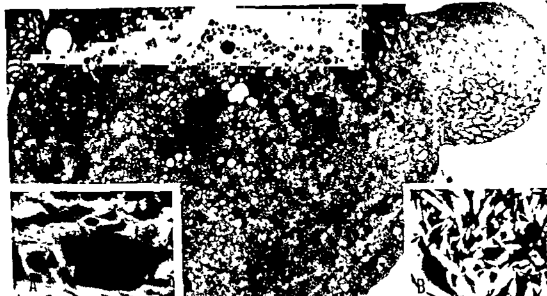
made of the borders of the three types of EH 118MG cell—the stellate and tumor giant cells fixed, prepared and cut while still attached to their culture vessel, i e *in situ* (see Materials and Methods), and the free, round EH 118MG cells, (the latter harvested from the culture medium) The stellate cells formed an interwoven mosaic whose cytoplasmic membranes were apposed but distinct and separate from each other No fusion was discerned The cytoplasmic surface of the free cells carried small, filiform projections or blunt protrusions (Fig 16) Again apposing membranes were intact and carried surface acid mucopolysaccharide

The plasmalemma of EH 118MG cells presented a unique feature, considered to be concerned with cytoplasmic fusion Stages of what was interpreted to be cell fusion in progress were observed at the surface of certain giant cells These carried numerous long slender, narrow based, externally projecting blebs or pseudopodia (Figs 17, 18) which interlocked with analogous protrusions from contiguous cells The junctional area between such interlocking processes was demarcated some times by two intact plasmalemmal membranes, i e no fusion was present In other cases, this junctional region devoid of intact cytoplasmic membranes was represented by a row of vesicles (Fig 17, single arrow) and/or a bundle of microfibrils (Fig 17 double arrow) It was in such instances that cytoplasmic fusion was considered to be a distinct possibility Stereomicroscopy of these areas confirmed the absence of intact cytoplasmic membranes The different texture or content of the cytoplasm of two such cells believed to have been in the act of fusion is seen in Fig 18 The other projecting protrusions already described in free EH 118MG cells (Fig 16) and subsequently in this report in murine tumor astrocytes (*MMG*) (*Fig 19*) were smaller stubbier, more broadly based and always covered by intact cytoplasmic membrane

7 *Murine tumor astrocytes* (MMG 1–5) (Figs 19–23) The most striking feature of these tumor cells was their abundance of virus particles (Figs 19–23) Sometimes the virus was present in the formative stage budding from the cisternal and cytoplasmic surfaces (Figs 21–23) Many other particles lay apparently free within the cisterns which were often distended (Fig 21), or external to the cell surface The presence of C type particles (*Dalton et al* 1966) and the positive immunodiffusion tests for MuLV sl (*Geering et al* 1966, *Nowinsky et al* 1968) confirmed the presence of murine leukemia virus Virus particles were seen both in cultured cells, and in solid tumor caused in mice by injection of these cells In the latter case, the particles were all intracisternal in sections examined The solid tumors also carried necrotic cells and areas of cytoplasmic vacuolation – features not seen in their progenitors in culture

The plasmalemmal surface of the MMG cells was in general unremarkable that is was continuous, intact and smooth with an occasional microvillus One exceptional cell group in culture (Fig 19) carried multiple broad based protrusions, similar to those frequently seen in Rous transformed tumor astrocytes (Fig 16) (*Macintyre et al* 1969)

Figs 19 23 Murine tumor astrocytes (MMG) Histologic section of a two month-old grid culture from a murine glioblastoma shows closely packed pleomorphic tumor astrocytes including one giant cell (Fig 19A) The murine tumor astrocytes which grew *in vitro* from this source (*Fig 19 B*) have similar morphologic features to the primary tumor (Fig 19A Hematoxylin and eosin Magn × 100 [illegible] Magn × 100) [illegible] inhibit [illegible] tumor astrocytes had numerous virus particles which are readily identified as polyhedral units within the cytoplasm even on low magnification (Fig 19) The particles commonly bud from the surface of the granular endoplasmic reticulum cisterns (Fig 21) and from the cell surfaces (Figs 19 23) The blunt protrusions from the surface of one tumor cell (Fig 19) are an unusual feature for this group and resemble those depicted in Fig 16 for free EH 118MG cells The rich cytoplasmic network of free microfibrils is best illustrated in Figs 20 and 21 (Fig 19 Magn × 11 000 Fig 20 Magn × 40 000 Fig 21 Magn × 30 000 Figs 22 23 Magn × 50 000)







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23

No virus particles were associated with these protrusions

The membrane system was impressive, especially the Golgi apparatus and its abundant vesicles An elaborate meshwork of intracytoplasmic microfibrils filled many areas Microtubules were present throughout the cytoplasm including the microfibrillar rich regions However, no special organization of microtubules in groups such as had been observed in the human tumor astrocytes was seen adjacent to the plasmalemma Ribosomes were often attached to membranes but by far the most common arrangement was the free polyribosome These were prominent in all the parts of the cytoplasm The cells phagocytosed carbon to the same extent as the other tumor astrocytes Abnormal mitochondria were not seen Occasional lipid like inclusions were found Lysosomes showed a variable development and represented all stages Giant cells were simply larger versions of the astrocytes described A thick surface layer of acid mucopolysaccharide was present

DISCUSSION

A problem inherent in electron microscopy is that the fraction of total material which can be examined is necessarily minute even with multiple sections and exhaustive viewing This introduces some bias in the information collected and the authors present their interpretations made after a three year study with these difficulties firmly in mind

Fibroblast in high density cultures had very dense mats of external microfibrils which were occasionally beaded (*Hayes & Allen* 1967) and were digested by collagenase This material was considered to be tropocollagen No specific banding was found but the cultures were not supplemented by vitamin C which is reported to be required for mature collagen production (*Green et al* 1966) External microfibrillar arrays were also found associated with astrocytes These were never dense never beaded and were resistant to collagenase They developed only after cell to cell contact had been established and became increasingly prominent with the duration of such contact They were considered to be equivalent to the fibrillary material described as present in minute amounts between contiguous normal adult astrocytes in culture at terminal cell density (*Pontén et al* 1969) Their chemical nature is unknown Astrocytes, especially in high density cultures are known to contain S 100 protein (*Benda et al* 1969), a protein reportedly unique to brain (*Moore & McGregor* 1965) The human tumor astrocytes 118MG do react with antiserum to this protein (kindly supplied by Dr *Levine*), but we have no evidence to associate this with the external microfibrils of the astrocytes whose composition remains obscure

Both benign and malignant human astrocytes in culture were usually extremely long slender spindles corresponding to the fibrous astrocyte of light microscopy Morphologic variants such as tumor astrocyte 118 D1 (Fig 5) derived from 118MG resembled the protoplasmic astrocyte of histology Intracytoplasmic gliofibrils which characterize both morphological types of astrocytes in whole tissue were demonstrated in the cultured astrocytes (benign and malignant) both after metallic impregnation (*Shein* 1965 *Pontén & Macintyre* 1968) in light microscopy and as varying sized bundles of microfibrils on electron microscopy They were especially prominent in the cytoplasm of the stellate form of RSV transformed human tumor astrocytes

The 119MG cell cultures included (in addition to the tumor astrocytes) cells which were apparently of antibody producing type The histologic and fine structural appearance (*Hummeler et al* 1966) of the rounded 119 MG cells suggested immunocytic capability and it has been reported (*Pontén & Macintyre* 1968) that cells from this culture did produce a clonal immunoglobulin (IgG)

Mitoses were found in vitro (on routine cytochemical stains from chromosome spreads and in the electron microscope) in all types of astrocytes confirming that mitosis is the mode of astrocyte replication in culture (Por

tén et al 1969) rather than amitosis (*Lumsden* 1968)

The apparently increased surface layer of acid mucopolysaccharides of malignant compared with normal cells supports the much more detailed studies of other workers in this field (*Defendi* & *Gasic* 1963)

The orientation of microtubules parallel to the long axis of the cells was a fairly constant feature of many astrocytes in culture Their intimate association with surface microfibrillary arrays was especially prominent in the 1181N1 and 105MG human tumor astrocytes We have not yet investigated the role of either of these structures in maintenance of cell form It has been reported that microtubule formations control the shape of certain lower forms (*Porter* 1966, *Tilney* 1968) It is tempting to postulate that microtubules function in a similar manner in these astrocytes However, an apparent contradiction to these hypotheses was seen when we compared stellate and round RSV-transformed human tumor astrocytes (EH 118MG) with the above spindle cells No parallel arrays of microtubular systems were found in either stellate or round EH 118MG cell types Furthermore, since round and stellate cells represent merely different morphological expressions of the same cell (*Macintyre et al* 1969), it was surprising that the microtubular arrangement within the two cell types was so similar despite the diversity of cell forms The only clearly distinguishing feature between these forms involved the microfibrillar system The cytoplasm of the stellate EH 118MG cells was traversed by a system of anastomosing microfibrillar bands, the arrays of which varied from 0 2 – 1 0 microns in diameter The round cells, on the other hand, lacked this complex and contained, very occasionally, paranuclear aggregates of microfibrils

The Rous cultures were also studied for evidence of the mechanism involved in cytoplasmic fusion and formation of giant cells Many cells closely packed in areas of high density, maintained the integrity of their plasmalemmal surfaces, and no fusion was detected by stereomicroscopy In less dense areas, irregular projections of contiguous cell surfaces could be discerned The base of these pseudopodial type extensions ranged from 5 – 20 microns Their length was much greater In many instances such processes were observed in intimate contact with those of neighbouring cells Examination of many such areas showed a few locations that were interpreted to represent fusion in progress between two cells This process was visualised as follows Subsequent to contact of the pseudopodial extensions of contiguous cells, the apposed portions of the plasmalemmas fused and in the initial stages, the original areas of contact persisted as a series of vesicles or small cisterns In instances this line demarcated by the vesicles was underlaid by a microfibrillar system Such microfibrillar arrays were frequently present just beneath the intact plasmalemma of nonapposed cell surfaces

The presence of C-type particles of the murine leukemia virus group in cultured murine tumor astrocytes derived from a methylcholanthrene induced primary tumor has a counterpart in Zimmerman's methylcholanthrene induced ependymoblastoma (malignant tumor of the ependymal cells of the brain, which line the ventricular system) (*Zimmerman* & *Arnold* 1941), in which virus particles have also been seen (*Rubin et al* 1969) The murine tumor astrocytes carry the murine leukemia virus group specific antigen, while Zimmerman's cells contain the murine mammary tumor virus group specific antigen (*Rubin et al* 1969, *Lloyd J Old*, personal communication) There has been much recent speculation that chemical carcinogens induce the development of pre-existing latent tumor viruses Our present report adds another instance where chemical carcinogen and murine tumor viruses appear to be associated

Wallach (1968) has stressed the importance of membrane defects in cancer cells He uses "membrane" in its widest possible sense, including formed structures as mitochondria lysosomes, the Golgi complex, granular endo-

plasmic reticulum as well as microfibrils, microtubules, nuclear and cytoplasmic membranes The morphologically abnormal mitochondria seen in certain human tumor astrocytes would fit this concept, as they were seen only in cancer cells A case could also be made that the marked hyperplasia of the *Golgi complex* in tumor astrocytes represented not functional hyperactivity (producing membranes, for example) but hyperplasia related to functional inefficiency The tumor lysosomes were functional (as judged by the presence of acid phosphatase), but the fact that they did not disappear in the logarithmic phase of cell growth (as those of the 125CG cells did) may represent a defect in the disposal mechanism (about which nothing is known in any cell) HeLa cells (*Gordon et al* 1965) follow the pattern of 125CG cells in this regard

These studies have underlined the fact that the morphological difference in structural details between normal and tumor derived astrocytes is in general quantitative rather than qualitative The functional integrity of the various membranes is another matter (*Wallach* 1968), and may be impaired as in other tumor cell systems

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This investigation was supported by grant NB 07983 from the *National Institute of Health U S A* *Damon Runyon Memorial Fund Jane Coffin Childs Memorial Fund, The Swedish Cancer Society the Swedish Medical Research Council* and *the Ollie Ericsson Foundation, Sweden* and *the Milheim Foundation U S A* The authors wish to thank *National Jewish Hospital and Research Center, Denver* for research facilities generously furnished

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BRIEF REPORTS

HEPATIC ALKALINE PHOSPHATASE ACTIVITY IN MALIGNANT DISEASES

I Hagerstrand

The alkaline phosphatase activity of normal human livers is histochemically localized to sinusoids periportally and centrolobularly and to the walls of the portal vessels and central veins (Fig 1) A canalicular activity in the human liver is abnormal It is sometimes seen in cirrhosis cholestasis hepatitis in the periphery of tumours (Aronsen et al 1969) This brief report describes the finding in some human liver biopsy specimens of normal histological

Fig 1 T 182/68 Malmoe Normal alkaline phosphatase reaction in liver tissue from a 26 years old man Portal zone down central vein up × 184

Received 5 i 72 from the *Institute of Pathology the Histochemical Department, Malmo General Hospital, University of Lund, Malmo Sweden*

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...phatase reaction ... tissue ...erated upon for a renal carcinoma (T 7625/71 Malmoe) Portal zone down × 184

appearance but with such canalicular activity (Fig 2) The patients were later found to have malignant diseases without involvement of the liver (renal carcinoma malignant lymphogranulomatosis adenocarcinoma without known origin) Thus if in a liver biopsy specimen of normal histological appearance alkaline phosphatase activity is demonstrable in canaliculi one should consider a malignant disease with or without hepatic involvement

Reference Aronsen K F *Hagerstrand I* & *Nordén J G* Acta Chir Scand *135* 619-624 1969

DEMONSTRATION OF GLYCOSAMINOGLYCANS IN FLUID FROM JAW CYSTS

Nils Skaug and *Tor Hofstad*

On the basis of total protein determination and paper electrophoresis *Toller* (8) concluded that fluid from cysts of the jaw was a simple dialysate from plasma He found albumin and β_1 globulins to be present in similar quantities as in serum β_2 globulins were not detected, and α_1 globulins were mostly absent α_2 globulins were found to occur more often but not in appreciable amounts The presence of IgG IgA and IgM in cyst fluid was demonstrated by immunoelectrophoresis (10)

The composition of fluid from 5 jaw cysts without clinical signs of inflammation or infection has been investigated None of the cysts were histopathologically classified as keratocysts The cyst fluids were sterile when aspirated and without visible blood con am na ... cyst fluids stored at were froze ... 20 C until used Serum from the same patient was always obtained and used as control and for comparison By electrophoresis on cellulose acetate membrane (CAM) (barbiturate buffer pH 8 6, ionic strength 0 06 current 0 5 mA/cm strip width) of cyst fluid the bands corresponding to proteins, lipopoteins and glycoproteins were more or less blurred Fig 1 (right) shows typical blurred protein bands in a sample of fluid from a large maxillary cyst. Pre-albumin albumin and γ globulins are present The albumin band has a characteristical drop shape and has a lower R_f value than serum albumin The disturbance of the electrophoretic protein separation was a constant finding although it was not equally pronounced with all cyst fluids examined By immu ...

sity due to its content of hyaluronic acid (HA), exhibits a similarly disturbed electrophoretic pattern (2) CAM-electrophoretograms of fluids from jaw cysts and of autologous sera were therefore stained with Alcian Blue (0 5 per cent aqueous solution, pH 4) which is specific for acid glycosaminoglycans (3), and periodic acid Schiff (PAS) Cyst fluids showed two coloured zones (Alcian Blue), whereas the autologous sera gave no staining (Fig 2) The fastest migrating factor was PAS negative Alcian Blue is usually used at pH 2 6, in 3 per cent acetic acid (4) At this low pH, masking of polysaccharide by protein may occur, however (5 7) In this investigation pH 4 was chosen to minimize this complication In order to depolymerize

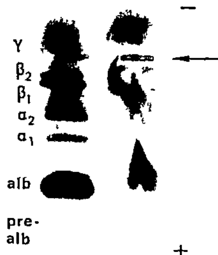


Fig 1 Protein CAM-electrophoretogram of patient serum (left) and autologous cyst fluid (right) Stained with 0 2 per cent amido black Arrow indicates point of application

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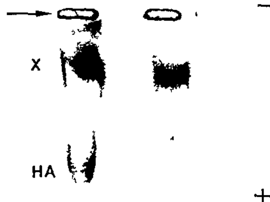


Fig 2 Acid glycosaminoglycan CAM electrophore [illegible]

(*right*) *Arrow indicates point of application* HA hyaluronic acid (?) x unidentified fraction

the glycosaminoglycans, thought to be hyaluronate, samples of cyst fluids were treated with bovine testicular hyaluronidase (Sigma, 820 NF units/mg, Lot 11C–0750) One ml of cyst fluid was incubated at 37° C for 2 hrs with 0 2 ml of a hyaluronidase solution containing 3 mg enzyme in 1 2 ml of 0 15 M NaCl On CAM electrophoresis of the

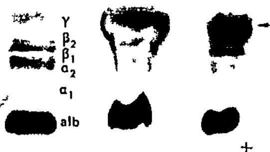


Fig 3 Protein CAM electrophoretogram Stain 0 2 *per cent* Ponceau S Patient serum (left) untreated cyst fluid (middle), hyaluronidase digested cyst fluid (right) Arrow indicates point of application

digested fluid, the proteins now showed a separation pattern similar to that of serum (Fig 3) The same was true with lipoproteins and glycoproteins. When stained with Alcian Blue, the digested cyst fluid gave only one coloured fraction (Fig 2) the fraction with the highest R_f-value having disappeared By disc electrophoresis in acrylamide gel cyst fluid treated with hyaluronidase was better separated than undigested samples

By immunodiffusion in agar, cyst fluid was shown to contain IgG, IgM, IgA and fibrinogen The results therefore suggest that fluid from jaw cysts contains the same main protein components as does plasma In addition, two different acid glycosaminoglycans appear to be present The fraction with the highest migration rate in CAM-electrophoretograms disappeared after treatment with hyaluronidase At the same time, the viscosity of the cyst fluid decreased Several investigators have *found that partly depolymerized HA in a purified* form reacts either weakly or not at all in the PAS *reaction (1) This indicates that the fraction which* disappeared in digested cyst fluid, may be HA (or *another glycosaminoglycan sensitive to treatment* with hyaluronidase) The slowly migrating factor *has not been identified*

Osmotic diffusion is one of the mechanisms whereby the growth or enlargement of cysts may be explained (6, 9), and the osmolarities of cyst fluids have been found to be higher than those of patients blood sera (6) HA would be expected to contribute to increased osmotic pressure

The origin of acid glycosaminoglycans in fluid from jaw cysts is still obscure They may be produced by cells in the cyst wall or they may be products from the osteolysis

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Acta path microbiol scand Section A 80, 287–288, 1972

EFFECT OF NEONATAL THYMECTOMY ON THE PRIMARY HAEMOLYSIN RESPONSE AND ON LYMPH NODE CELL COUNT IN FIVE STRAINS OF RATS

Kirstine Borum

Neonatal thymectomy in mice causes a depression of certain immune functions, among these the primary haemolysin response following an injection of sheep erythrocytes (1, 2, 3) In rats the situation is more complex *Pinnas & Fitch* (4) found in neonatally thymectomized and non thymectomized CFN rats given sheep erythrocytes at the age of 4 weeks, an equal response as measured by the number of haemolysin forming cells of spleen and lymph nodes In our laboratory, however, a reduction of the serum haemolysin titres measured five days after the injection of sheep erythrocytes intraperitoneally was consistently found in three months old Sprague Dawley rats (not inbred, Anticimex, Stockholm) when thymectomy was performed within the first 24 hours after birth as compared to sham-operation Also the number of cells in regional lymph nodes (the left group of parathymic lymph nodes) was smaller in neonatally thymectomized rats than in the sham-operated controls Results of a typical experiment is listed in Table 1

Further studies in the research program implicated the transfer of lymphoid cells from one animal to the other therefore it was found desirable to obtain an inbred rat strain for these experiments The inbred white Amsterdam rat strain was available and neonatal thymectomy and sham operation was performed on a large number of animals When three months old the rats were challenged with sheep erythrocytes and their serum haemolysin titres were measured From unstimulated thymectomized and sham-operated rats the cell content of the left parathymic lymph nodes was determined No effect of neonatal thymectomy however was found upon the primary serum haemolysin titre or upon the lymph node cell count as compared to sham-operation A representative experiment is presented in Table 2

Received 20.xii 71 *from the Department of Pathology University Hospital, 221 85 Lund, Sweden*

As a consequence of these results it was decided to examine the influence of neonatal thymectomy upon the primary haemolysin response and on lymph node cell count in three other inbred strains of rats namely the Wistar/Fu strain, the Black/Norway strain and the Fisher strain Controls were non-operated litter mates When the animals were three months old, 0 1 ml of a 2 per cent solution of sheep erythrocytes was injected into each of the two hind foot pads Five days later the animals were bled and their serum haemolysin titres determined and as in the earlier experiments, expressed as the negative 2 logarithm to the dilution of the serum which gave 50 per cent haemolysis Cell counts of the two pooled popliteal lymph nodes from each rat were also determined The results are seen in Table 3

Different rat strains apparently react differently to neonatal thymectomy The inbred Amsterdam strain did not react at all In this strain aberrant thymic tissue was looked for macroscopically, but not found The greatest influence of neonatal thymectomy upon the primary haemolysin response to sheep erythrocytes was encountered in the inbred Wistar/Fu strain, so this strain was chosen for further experiments on the influence of thymectomy upon other immune responses (5) It is suggested that the conflicting results of the influence of neonatal thymectomy in the rat are due to strain differences

This investigation was supported from the *Swedish Medical Research Council*

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TABLE 1 *Serum Haemolysin Titres Following the Injection of 1 ml/100 g of Body Weight of a 0 2 per cent Suspension of Sheep Erythrocytes Intraperitoneally, and Regional Lymph Node Cell Counts in Neonatally Thymectomized and Sham-Operated Sprague Dawley Rats*

	Number of rats	Average serum haemolysin titre	Average lymph node cell count
Thymectomized ♂	6	3 8	5 3 $\times 10^6$
Sham operated ♂	6	6 2	20 1 $\times 10^6$
Thymectomized ♀	4	4 6	6 0 $\times 10^6$
Sham operated ♀	4	6 5	14 5 $\times 10^6$

TABLE 2 *Serum Haemolysin Titres Following the Injection of 1 ml/100 g of Body Weight of a 0 2 per cent Suspension of Sheep Erythrocytes Intraperitoneally into Neonatally Thymectomized and Sham Operated Inbred Amsterdam Rats Cell Counts of Parathymic Lymph Nodes from Unstimulated Rats Are also Listed*

	Number of rats	Average serum haemolysin titre	Average lymph node cell count
Thymectomized ♂	4	6 7	16 0 $\times 10^6$
Sham-operated ♂	4	6 8	10 2 $\times 10^6$
Thymectomized ♀	3	6 8	1 9 $\times 10^6$
Sham operated ♀	3	6 8	2 4 $\times 10^6$

TABLE 3 *Serum Haemolysin Titres Following the Injection of Sheep Erythrocytes 0 1 ml of a 2 per cent Solution, into the Hind Foot Pads, and Cell Counts of Popliteal Lymph Nodes, in Neonatally Thymectomized and Non Operated Rats of Three Different Inbred Strains*

Rat strain		Number of rats	Average serum haemolysin titre	Average cell count of right + left popliteal lymph nodes
Wistar/Fu	Thymectomized ♂	5	1 2	9 5 $\times 10^7$
	Non-operated ♂	5	5 5	4 2 $\times 10^6$
	Thymectomized ♀	4	2 0	1 7 $\times 10^6$
	Non-operated ♀	6	5 7	5 2 $\times 10^6$
Black/ Norway	Thymectomized ♂	4	3 7	7 6 $\times 10^6$
	Non operated ♂	5	5 7	2 0 $\times 10^7$
	Thymectomized ♀	4	3 7	8 2 $\times 10^6$
	Non-operated ♀	5	5 2	1 1 $\times 10^7$
Fisher	Thymectomized ♂	6	0 5	2 8 $\times 10^6$
	Non-operated ♂	6	1 7	3 5 $\times 10^6$
	Thymectomized ♀	6	1 9	2 3 $\times 10^6$
	Non-operated ♀	6	3 8	5 1 $\times 10^6$

Acta path microbiol scand Section A 80 289-293, 1972

MARGINAL VACUOLES IN FINE NEEDLE ASPIRATION BIOPSY SMEARS OF TOXIC GOITERS

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A special type of vacuoles called marginal vacuoles and occurring in the cytoplasm of the follicular epithelium cells in smears from fine needle aspiration biopsy specimens is described Marginal vacuoles were found to be much more frequent in toxic than in atoxic goiters The difference was so clear that it was considered to be of some value in the examination of biopsy smears from the thyroid It is suggested that markedly dilated cisterns of the endoplasmatic reticulum are the most likely ultrastructural counterparts of the vacuoles

Reports about features distinguishing between toxic and atoxic goiters, as reflected in the cytological picture of biopsy smears from fine needle aspirates are rare *Soderstrom* (1952) found so called paravacuolar granulations to be more numerous in the cytoplasm of the follicular epithelium in toxic than in atoxic goiters However, he felt this finding to be of very limited differential diagnostic value (*Söderström* 1966) Differences between toxic and atoxic goiters regarding nuclear size and cytoplasm content of granules and vacuoles have also been investigated by *Myren et al* (1962) They reported a larger nuclear size and a larger number of paravacuolar granulations in toxic than in atoxic goiters Paravacuolar granulations were noted in one third of the toxic goiters

This paper concerns a special type of vacuoles in the follicular epithelium of goiter aspirates—a type thought to be of interest in the differential diagnosis between toxic and atoxic goiters as well as in the physiology of secretion by these goiters These vacuoles were called marginal vacuoles (MV) because they were usually situated peripherally in the groups of follicular epithelium formed in smears from goiter aspirates

MATERIAL AND METHODS

The material consisted of 49 cases of untreated toxic goiter and 35 cases of benign atoxic goiter without signs of thyroiditis In both groups the cases were selected consecutively from the goiters subjected to fine needle aspiration biopsies in the medical department of Lund after exclusion of a few biopsy specimens which at preliminary examination were considered to be too poor in cells for the present investigation The diagnosis of hyperthyroidism or euthyroidism was supported by clinical and laboratory examinations, including protein bound iodine (PBI) in serum and/or T_3 uptake test In 2 cases classified as atoxic goiters there was a slight elevation of PBI as judged by the criteria used at the central laboratory of the hospital However a normal T_3 uptake as well as the clinical picture suggested atoxic goiters in both cases In 44 *cases of toxic goiters and 15 cases of atoxic goiters* radio-iodine uptake was determined and lent support to the definitive diagnosis In all cases of toxic goiters the long acting thyroid stimulating

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factor, LATS, was determined by means of a modification of the method described by *McKenzie et al* This determination was made in the department of Pharmacology of the University of Lund LATS was demonstrated in 14 cases

All the biopsies were made with a fine needle with an outer diameter of 0 6–0 7 mm The biopsy technique has been described in detail by *Soderstrom* (1966) After the smears had been air dried they were stained with the May–Grunwald Giemsa (MGG) technique Some specimens were also stained for periodic acid schiff reaction (PAS) and for acid phosphatases by means of the simultaneous azo dye coupling method using naphtol AS B_1 (*Burstone* 1958)

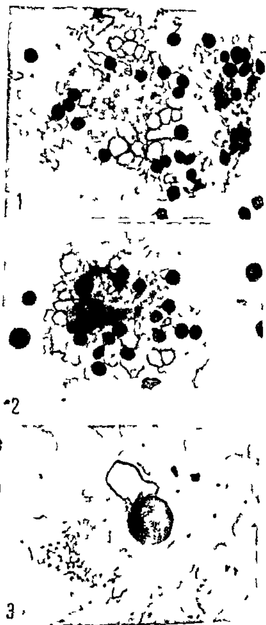
For comparison also wet fixed haematoxylin-eosin stained smears were examined in 10 cases of toxic goiter Furthermore histological sections from surgical specimens of eight toxic goiters were examined as well as aspirate smears and imprints from the same specimens These specimens were taken after pre operative treatment with carbimazol and 1 thyroxin

RESULTS

The follicular epithelium of MGG stained biopsy smears from the toxic goiters were usually very rich in cytoplasmic vacuoles which ranged from a barely visible size to giant vacuoles exceeding nuclear size (Figs 1–3) Most of these vacuoles were of irregular outline Their content was homogenous and stained slightly pink with MGG especially in the peripheral part of the vacuoles They were usually found in combination with a pale large nucleus They tended to gather in the peripheral part of the cell groups formed in biopsy smears Because of the latter finding *they were referred to as marginal vacuoles* (MV)

MV could be easily distinguished from the vacuoles associated with paravacuolar granulations (*Söderström* 1952, *Myren et al* 1962) by their lack of vacuole bound granules but they could not be clearly separated from other rarely occurring vacuoles without the marginal position and the irregular contour characteristic for MV

The marginal vacuoles stained poorly or not at all with PAS They were not positive for acid phosphatases In wet fixed haematoxylin-eosin stained smears routinely used



Figs 1 3 Marginal vacuoles from six cases of toxic goiter
Figs 1 2 160 × and fig 3 400 ×

in many laboratories MV were not as distinct as in dry fixed MGG stained smears In histological sections from surgical specimens stained with haematoxylin-eosin MV could not be identified with certainly even when aspirate smears from the same specimen revealed distinct MV *Imprints from these specimens* were found to be much poorer in MV

TABLE 1 *Occurrence of Marginal Vacuoles (MV) in Different Types of Fine Needle Aspirates from the Thyroid Gland*

	Abundant MV	Moderate MV	Scanty or none MV
Toxic goiter	21	19	9
Nontoxic goiter	1	10	24

The difference between toxic and atoxic goiter after pooling of the groups moderate and scanty or none MV is statistically significant ($p < 0.001$)

than aspirate smears The cytoplasm in imprints often appeared interrupted with numerous nuclei devoid of visible cytoplasm

The occurrence of MV was graded as follows

1 None or scanty Distinct MV exceeding 2 µ in diameter were demonstrated in no or only a few cells
2 Moderate MV were frequently seen but only in a minority of the cells examined
3 Abundant Presence of MV in a majority of the cells examined

At least 500 cells were examined in each biopsy specimen In order to avoid subjective bias the biopsy smears were coded before examination and read blind The results are given in Table 1 As is evident from this table MV were much more common in toxic than in nontoxic goiters In the group of toxic goiters the frequency of MV showed no statistically significant correlation with the results of PBI and T_3 test, the product of the values for PBI and T_3 (free thyroxine index) radio iodine uptake at 2 and 24 hours the quotient between the radio iodine uptake at 2 and 24 hours or demonstrable LATS-activity Nor was any correlation found between the occurrence of MV and age or sex of the patients in the toxic or atoxic goiter group

DISCUSSION

The difference between the occurrence of MV in toxic and atoxic goiters was according to the present investigation striking but not always clear enough to discriminate between the two types of goiters However, when MV are abundant they strongly suggest the presence of hyperthyroidism and may therefore be of value in the interpretation of biopsy smears from the thyroid However, the diagnostic value seems smaller in wet fixed smears stained with haematoxylin eosin and non existent in formalin fixed specimens treated with histological techniques

The occurrence of MV may at least partly, be due to technical factors Thus otherwise invisible vacuoles may expand owing to the very low pressure that has been demonstrated in the syringe during aspiration biopsies (*Cri born et al* 1964) This may explain the larger number of MV in aspirates than in imprints of surgical specimens Furthermore, in imprints of surgical specimens the cells are often very poor in cytoplasm suggesting that parts of the cytoplasm containing MV may have been torn off in this type of preparations

Ultrastructural studies of toxic goiters (*Irvine et al* 1963 *Heimann* 1966) have revealed structures which judging from their form and size may be counterparts of MV

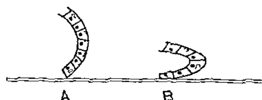


Fig 4 Diagram showing how flattening of follicle fragment may project the basal part of some cells containing markedly dilated cisterns of endoplasmatic reticulum free from other cell constituents A before flattening B after flattening



Fig 5 Flaming plasma cell from an IgA myeloma In the periphery of the cytoplasm structures closely resembl ng marginal vacuoles of thyro d follicular ep thel um can be ident f ed 400 ×

The most important of these are the cisterns of the endoplasmic reticulum (ER) *Hei mann* (1966) reported that such cisterns with a diameter of several microns and thus well within the light microscopical range can be found in toxic goiters Like MV these cisterns ar usually irregular in shape The lar gest cisterns are situated basally in the cells of the follicular epithelium—a finding which may correspond to the peripheral position of MV in the cell groups of aspirate smears Thus basal parts of some cells containing dilated cisterns may be projected free from other parts of the cells owing to the flattening of follicle fragments in smears This is illustrated in Fig 4 Furthermore in dry fixed smears the cytoplasm in individual cells is flattened and thereby spread over a comparatively *large area which facilitates recognition of* cytoplasmic details such as the cisterns of ER These possibilities would help to explain why MV are more prominent in dry fixed than in wet fixed smears where follicle fragments and individual cells are not flattened to the same extent

The assumption of a correspondence be tween MV and the cisterns of ER is also strengthened by observations of distinct MV in imprints and aspirates from rat thyroids during stimulation with thyrotropin or propylthiouracil for some weeks (Unpublished personal observations) Such stimulation is known to induce enlargement of the ER cisterns up to a light microscopical range (*Wissig* 1964 *Lupulescu et al* 1968) The endoplasmic reticulum and thereby probably MV, is known to play a central role in the formation of protein in the follicular epi thelium (*Nadler et al* 1964 *Ekholm et al* 1966) It is therefore reasonable to assume that MV are connected with the increased rate of protein synthesis probably occurring in toxic glands analogous to the findings in stimulated rat thyroids (*Debons et al* 196? *Raghupathy et al* 1963, *Tong* 1964)

In the author s experience structures re sembling MV in the follicular epithelium are rarely found in cells from other organs A striking exception is plasma cells and especial ly some types of myeloma cells which are called flaming cells (*Undrit*. 1952) because the cytoplasm stains red with the May Grun wald Giemsa technique The periphery of the cytoplasm in myeloma cells often contains structures with a remarkable resemblance to the marginal vacuolers described in this paper (Fig 5) It is noteworthy that marked en largement of the cisterns of ER has been noted in many myeloma cells and especially in the above mentioned flaming cells (*So rensson* 1964 *Maldonado et al* 1965 1966)

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Acta path microbiol scand Section A 80, 294-302, 1972

THE OCCURRENCE AND SIGNIFICANCE OF ABNORMAL BILE DUCT EPITHELIUM IN CHRONIC AGGRESSIVE HEPATITIS

A Comparative Morphological, Biochemical, Immunological, and Prognostic Study

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A material of 57 primary biopsies exhibiting chronic aggressive hepatitis from 57 patients has been selected from a total consecutive liver biopsy material of 3000 The material is divided into two groups group 1 21 patients with abnormal biliary epithelium, group 2 36 patients without abnormal biliary epithelium No morphological (apart from the atypical epithelium) or biochemical differences between the two groups have been demonstrated On the other hand the patients in group 1 are older (all over 45 years of age) than in group 2, just as there is a more pronounced excess of women and a more frequently insidious development of the disease There is significantly higher incidence of organ non specific antinuclear factors in group 1 than in group 2, whereas mitochondrial antibodies only is found in serum from one patient None of the available sera from group 1 and from the female patients in group 2 show presence of Australian antigen but in most of the male patients from group 2 precipitating lines are demonstrated A morphological follow up of slightly more than half of the patients shows a significantly more frequent development of cirrhosis in the group with abnormal biliary epithelium

Recently *Poulsen & Christoffersen* (11) have demonstrated abnormal bile duct epithelium in 14 cases of a consecutive series of 83 biopsies, all showing typical changes of the parenchyma as in viral hepatitis, some with acute and some with chronic hepatitis (6) A correlation with the biochemical and clinical findings showed that the incidence of abnormal bile duct epithelium is much more frequent in chronic hepatitis than in acute (5)

The purpose of this work has been to investigate how frequently atypical bile duct epithelium is encountered in a consecutive series of biopsies with changes as seen in chronic aggressive hepatitis (6) and whether there possibly are other morphological differences between liver biopsies with chronic

Received 9 ix 71

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aggressive hepatitis with and without atypical bile duct epithelium

The purpose has further been to make a comparison between a number of clinical, biochemical, and immunological parameters from the corresponding groups of patients and to examine the frequency of development into cirrhosis by assessment of repeat biopsies

MATERIAL AND METHODS

The material consists of a total of 57 percutaneous liver biopsies (from 57 patients) all with histologically verified chronic aggressive hepatitis (6) Cases morphologically giving suspicion of cirrhosis have been excluded and so have cases with acute viral hepatitis with slight or moderate periportal changes in the form of piece meal necroses

The biopsies have been selected as consecutive primary biopsies from a total of 3000 percutaneous liver biopsies received at the Pathological Anatomical Institute, Kommunehospitalet from seven medical departments in Copenhagen in the period December 1965–May 1970

The tissue has been fixed in neutral formalin and embedded in paraffin From the first 5 biopsies 10–15 sections were cut on a sledge microtome, while the last 52 biopsies were cut on a rotary microtome (40–50 sections)

The assessment has been performed without knowledge of the clinical data by two of the authors (P C and H P) in close cooperation on haematoxylin and eosin and v Gieson Hansen preparations For all biopsies further sections stained for reticulum fibres (8), iron (10), and pyroninophil substance (3) have been available

In addition to recording the occurrence of bile ducts with abnormal epithelium the following qualities have been semiquantitatively registered (0–3) focal lytic necroses confluent necroses piece meal necroses variation in size of liver cells and nuclei acidophilic bodies Kupffer cell proliferation cholestasis (non-controversial intra and extracellular bile thrombi) adenomatous liver cell proliferation fatty change parenchymal inflammation, passive septa (2) as well as iron and lipofuscin deposits in liver cells Kupffer cells and connective tissue

Further degree of bile duct proliferation fibrosis and inflammation in portal tracts have been registered

The material is divided into two groups *Group 1* containing bile ducts with abnormal epithelium and *group 2* without abnormal bile duct epithelium The histologic, clinical biochemical, and immunological features in the two groups have been compared

The *clinical* data have been based on particulars from the case record at the time of admission at which the biopsy was performed, from subsequent admissions, and from out patient controls

At the time of biopsy the biochemical tests mentioned in Tables 4 and 5 were performed Laboratory examinations were made by conventional methods, fractionation of serum proteins being made by paper-electrophoresis When different units for the laboratory tests were in use, they were recalculated to a common standard

From some of the patients sera drawn simultaneously with the biopsies were available for *immunological* studies

The indirect double layer immunofluorescent technique was used for the determination of the following auto antibodies anti nuclear factors (ANF), smooth muscle antibodies (SMA), thyroid cytoplasmic antibodies (TCA), parietal cell antibodies (PCA) and mitochondrial antibodies (MA) Cryostate sections of human thyroid gland human gastric mucosa and human kidney tissue derived from patients with blood group O were used as antigens for the organic non specific anti nuclear factors and for the cytoplasmic antibodies. The arterial walls in the tissues and the lamina muscularis of the gastric tissue were used as antigen for the SMA Blood smears prepared as described by *Wiik* & *Munthe* (15) and isolated hepatocytes from rat liver prepared as described by *Millman et al* (9) were used as antigens for the possible presence of granulocyte and liver specific anti nuclear factors respectively

Sera were tested undiluted except when SMA were determined where a dilution of 1 10 was employed. FITC labelled anti human globulin reagents were prepared from heavy chain specific rabbit antihuman IgG and IgM sera (Nordic Netherlands) by reacting the globulin fraction precipitated with 50 per cent saturated ammonium sulphate with fluorescein isothiocyanate at 20 μg/mg protein for 30 minutes at pH 9 5 and 25°C Unreacted dye was removed by filtration through Sephadex G25 The class specific reactivity of the antisera was confirmed by immunoelectrophoresis The specificity of the corresponding conjugates was tested on marrow specimens from patients with IgG myeloma and Waldenstroms disease The microscope used was a Leitz Zernicke microscope equipped with a Tioyda darkfield carcoid condensor An iodine quartz lamp was used together with a primary interference filter, 495 nm with a high transmission of light (12) and a secondary

A M Prince and Dr *R W McCollum* as earlier described (7)

On 32 patients *repeat biopsies* have been performed. These biopsies have been assessed according to the same criteria as the primary biopsies. The average period of observation has been ten months varying from one month to 42 months. Nine patients have received steroids and one both steroids and immunosuppressive treatment.

A comparison has been performed between histologic features in two new groups (1 A and 2 A) which consist of the repeat biopsies from patients belonging to group 1 and 2.

For the statistical assessment the chi² test has been used. The limit for type 1 error (2 α) has been set to 0.05.

RESULTS

Morphological findings. Atypical bile duct epithelium is found in 21 (37 per cent) of the 57 biopsies (group 1), whereas 36 (63 per cent) are without atypical epithelium.



Fig. 1. Bile duct with abnormal epithelium in approximately one half of the circumference. The epithelium is here multilayered and the cells polygonal, irregular with light, empty looking cytoplasm. The circumference of the bile duct is indicated by arrows. × 250.

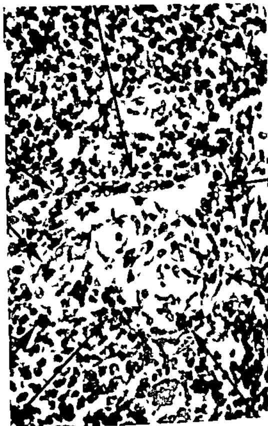


Fig. 2. As Fig. 1. × 250.

The main histologic changes observed in the affected portal bile ducts are most often multilayered epithelium with swollen cells with pale, lightly coloured often "empty looking" or vacuolated cytoplasm (Figs. 1 & 2). Often the cells are partly confluent with more or less ill defined cellular limits. The nucleus may show karyopyknosis and/or karyorrhexis. Generally there are areas with an indistinct basement membrane. The bile duct epithelium and the basement membrane are frequently infiltrated with lymphocytes and sometimes plasma cells and granulocytes. The lumen may contain cellular debris and may be partly obliterated (Fig. 3).

In the vicinity there is a considerable infiltration with lymphocytes, histiocytes and plasma cells. Only in a few cases signs of development of germinal centres are observed.

Serial sections show that the changes are segmental and often only embrace part of



Fig 3 Bile duct with abnormal epithelium affecting the whole circumference The epithelium is multilayered and the cells enlarged with granular or vacuolated cytoplasm The lumen contains cellular debris and in the surroundings a dense cellular infiltrate is seen The circumference is indicated by arrows × 156

the circumference of the bile duct (Fig 1) In each biopsy only one, or possible two or three, abnormal bile ducts are seen

Serial sections too show that the lesion has in all cases affected ducts of medium size with a central position in the portal areas Bile ducts with abnormal epithelium are frequently but not always situated in close relation to moderate or heavy infiltrates of inflammatory cells and all infiltrates should in our experience be examined very carefully as to whether a bile duct with abnormal epithelium is hidden among the many cells

A summary of the *parenchymal and portal changes* in the two groups are shown in Tables 1 and 2 As it appears from the tables, there is no statistically significant difference between the two groups neither as regards incidence nor degree of the registered histologic features

Clinical Findings

Group 1 comprises 17 women and four men They are all over 45 years of age, and the average age is 64 and 72 respectively (Table 3)

Group 2 comprises 23 women and 13 men The average age for women is 54 years and for men 29 years (Table 3)

As further seen from Table 3 the onset of illness was acute (debuting as a clinical acute hepatitis) in ten cases from group 1 and in 29 cases from group 2

Statistically there are significantly more cases with insidious onset of illness in group 1 than in group 2 ($p < 0.05$)

Biochemical Findings

The average values for some conventional liver tests can be seen in Table 4 The values were of the same order of magnitude in both groups, but the scatter of the values within each group is too great to allow a statistical comparison

As shown in Table 5 the incidence of hypoalbuminemia and hypergammaglobulinemia respectively are nearly the same, and there are no statistical differences between the groups

Immunological Findings

The results of the immunological findings are seen from Table 6 A high incidence (56 per cent) of organ-non specific anti nuclear factors was seen, but significantly more often in group 1 than in group 2 ($p < 0.05$) More female than male patients contained ANF in their sera No definite organ-specific anti nuclear factors were found in the present material

Sixteen out of 27 sera contained smooth muscle antibody, but the difference in the two groups are of no significance The SMA were of the IgG class and most of the sera also contained IgM antibodies, but in no sera was IgM antibody to smooth muscles identified in the abscence of an IgG antibody Four of the SMA in group 1 showed a pattern distinguishable from those previously described and resembled the zebra like fluorescence caused by striated muscle antibodies sometimes seen in myasthenia gravis (13)

Only one out of 39 patients sera tested showed mitochondrial antibodies and two

TABLE 1 *Number of Biopsies with the Following Parenchymal Changes in Group 1 (21 Biopsies with Atypical Bile Duct Epithelium) and Group 2 (36 Biopsies without Atypical Epithelium)*

Parenchymal changes	Group 1				Group 2				Difference between group 1 and 2 (chi² test)
		+	++	+++		+	++	+++	
Lytic necroses	21	18	3	–	36	31	5	–	no significance
Confluent necroses	5	3	2	–	8	6	2	–	no significance
Passive septa	6	6	–	–	13	11	2	–	no significance
Piece meal necroses	21	16	4	1	36	22	14	–	no significance
Acidophilic Bodies	21	21	–	–	36	32	4	–	no significance
Variation of cells and nuclei	21	14	6	1	36	22	14	–	no significance
Fatty change	6	6	–	–	14	13	1	–	no significance
Kupffer cell proliferation	21	13	6	2	36	15	19	2	no significance
Adenomatous proliferation	8	7	1	–	16	14	2	–	no significance
Cholestasis	6	5	1	–	12	12	–	–	no significance
Parenchymal inflammation	9	8	1	–	17	17	–	–	no significance
Lipofuscin in liver cells	21	19	1	1	36	33	3	–	no significance
Iron in liver cells	2	2	–	–	2	2	–	–	no signifi
Iron in Kupffer cells	6	5	1	–	7	3	4	–	no signific

TABLE 2 *Number of Biopsies with Following Portal Changes in Group 1 and 2*

Portal changes	Group 1				Group 2				Differen between g 1 and (chi² tes
		+	++	+++		+	++	+++	
[illegible]	21	12	9	–	36	20	16		no signific
[illegible] ion	21	5	6	10	36	9	15	12	no signific
[illegible] proliferation	21	10	11	–	36	22	13	1	no signific
Iron in portal macrophages	6	5	1	–	5	3	2	–	no significa
Germinal centres	3	3	–		1	1	–		no significa

TABLE 3 *Distribution of Patients in Group 1 (21 Patients with Abnormal Epithelium) and Group 2 (36 Patients without Abnormal Epithelium) According to Sex, Average Age and Onset of Illness*

	Male		Female		Onset of illness	
	Number	Average age	Number	Average age	Acute	Insidious
Group 1	4	72 (46–89)	17	64 (45–81)	10	11
Group 2	13	29 (15–64)	23	54 (19–78)	29	7

TABLE 4 *Average of Routine Liver Tests in Group 1 and 2*

	Group 1 (Patients with abnormal biliary epithelium)	Group 2 (Patients with normal biliary epithelium)
Serum bilirubin (< 1 0 mg/100 ml)	3 0 (0 4–10 9)	2 9 (0 3 10 5)
Serum GO Transaminase (< 34 U/L)	311 (58–1480)	269 (22–1166)
Alkaline phosphatase (< 74 U/L)	216 (47– 755)	194 (53–1073)

Normal values are given in parentheses below the name of the laboratory test

TABLE 5 *Number of Patients with Hypo albuminaemia and Hyper gammaglobulinaemia in Group 1 and 2*

	Group 1 (Patients with abnormal biliary epithelium)	Group 2 (Patients with normal biliary epithelium)	Difference between group 1 and 2 (chi² test)
Number of patients with hypo albuminaemia	10 (48 per cent)	17 (47 per cent)	no significance
Number of patients with hyper gammaglobulinaemia	17 (81 per cent)	23 (62 per cent)	no significance

TABLE 6 *Results of Immunological Tests*

Test	% positive in 50 controls	Group 1		Group 2		Difference betwe group 1 and 2 (chi² test)
		Number of patients examined	Number of patients with positive sera	Number of patients examined	Number of patients with positive sera	
ANF	2 %	13	11 (85 %)	26	11 (42 %)	$p < 0\ 05$
SMA	8 %	8	6 (75 %)	19	10 (52 %)	no significance
TCA	6 %	13	2 (15 %)	26	2 (8 %)	no significance
PCA	4 %	8	0	19	0	no significance
MA	2 %	13	1	26	0	no significance
Au ag	0 %	8	0	19	7 (37 %)	no significance

(ANF = anti nuclear factor SMA – smooth muscle antibody, TCA = thyroid cytoplasmic anti body, PCA = parietal cell antibody, MA = mitochondrial antibody Au ag = Australian antigen)

sera from each of the two groups contained antibodies to thyroid cytoplasma, no sera stained gastric parietal cells

With respect to identification of Australian antigen none of the 8 available sera from group 1 showed presence of the antigen, but 7 of the tested 19 sera from group 2 showed precipitating lines All these 7 sera were derived from male patients, and none of the 11 female patient sera contained the antigen

Follow up

Table 7 shows the distribution according to chief histological diagnosis of the 13 repeat biopsies in group 1 A and the 19 repeat biopsies from group 2 A

TABLE 7 *The Table Shows the Distribution of Repeat Biopsies According to Histological Diagnosis*

Chief histological diagnosis	Group 1 (repeat biopsies from 13 patients)	Group 2 (repeat biopsies from 19 patients)
Cirrhosis	7 (54 %)	2 (11 %)
Suspicion of cirrhosis	1 (8 %)	–
Chronic aggressive hepatitis	5 (38 %)	12 (63 %)
Chronic persistens hepatitis	–	1 (5 %)
Liver with portal fibrosis	–	1 (5 %)
Liver without pathological changes	–	3 (16 %)

Seven cases (54 per cent) from 1 A developed cirrhosis, and in one case there is suspicion of cirrhosis The last five cases from group 1 A still show chronic aggressive hepatitis *whereas no biopsies showed histologic improvement* In eight of the repeat biopsies (62 per cent) bile ducts with atypical epithelium were demonstrated (five of the cases with cirrhosis and three of the cases with chronic aggressive hepatitis)

Twelve (63 per cent) of the 19 biopsies from group 2 A still show aggressive hepatitis Two cases (11 per cent) have developed cirrhosis and two biopsies show respectively chronic persistent hepatitis and liver with portal fibrosis Three biopsies from this group show normal liver tissue In four cases (21 per cent) bile ducts with atypical epithelium were demonstrated (two of the cases with chronic aggressive hepatitis and the two cases with cirrhosis) *Abnormal bile duct epithelium has thus been demonstrated in all the cases which during the period of observation which was 10 months for both groups developed cirrhosis*

There are significantly more biopsies with cirrhosis in group 1 A than in group 2 A ($p < 0.01$)

Five patients in group 1 A have been treated with steroids Four of these still exhibited chronic aggressive hepatitis in the repeat biopsies, while one developed cirrhosis Also five patients in group 2 A were treated with steroids In all repeat biopsies chronic aggressive hepatitis was again demonstrated

DISCUSSION

As it appears from the results, chronic aggressive hepatitis is found in 57 primary biopsies of the 3,000 biopsies examined To the authors knowledge no comparable surveys exist As the composition of biopsy materials is more or less variable, comparisons between different materials of this kind are of limited value It must however, be noted that the figures given are minimal values since all cases, which morphologically give suspicion of cirrhosis have been excluded just as cases with acute viral hepatitis with slight or moderate portal and periportal changes with fibrosis and piece meal necrosis have been excluded It must therefore be justified to claim that chronic aggressive hepatitis in our material which is derived from a Scandinavian metropolitan area is not exceedingly rare

It further appears that approximately one third of the biopsies with chronic aggressive hepatitis concurrently exhibit atypical bile duct epithelium Apart from atypical epithelium no other registered differences between the two groups are found and especially no differences in the degree of fibrosis and activity have been demonstrated It might be expected that even though no histological differences were found between the whole of group 1 and the whole of group 2 that there could be differences between the 8 primary biopsies from group 1 A which later developed cirrhosis and the 5 from group 2 A which subsequently exhibited partial or complete regression These primary biopsies have

been compared histologically both with the respective groups and mutually, and it has not been possible to demonstrate differences

It is surprising that abnormal biliary epithelium has been found in more than one third of all cases with chronic aggressive hepatitis, when it previously only has been described in very few cases (1, 14) It seems to us, that there can be no doubt, that it is our extensive use of serial sections, that determines the difference, as the changes, as already noted, are segmental

As it appears from the results, only few differences between the two groups as regards clinical and biochemical findings have been demonstrated Atypical biliary epithelium has thus only been found in patients over 45 years of age and quite predominantly in women, just as the disease much more frequently develops insidiously in the group with abnormal biliary epithelium

The total incidence of Au antigenemia in the present material is 25 per cent and is thus in accordance with some previous reports (4, 15, 17), but the different frequency for the two groups in the present report (0–37 per cent) is remarkable, as is the presence of Au ag in the sera from male patients only The latter finding confirms that of *Bulkley et al* (4) also in the respect that none of the Au ag positive sera contained ANF as well Three of the 7 Au ag positive sera also showed SMA which is in contrast to the findings of *Visher* (16), who reported a mutual exclusion between Au antigenemia and presence of SMA The explanation for this discrepancy might well be the different dilution of the sera used in the two reports *Visher* only regarded titres 1 40 positive for SMA, this involved that none of the normal controls were positive When in the present study a dilution of 1 10 was employed 8 per cent of a control material matched with respect to age and sex showed positive reactions In Visher s series of 110 chronic hepatitis cases 10 5 per cent of the sera also contained mitochondrial antibodies, and neither of these sera contained Au ag In the present report only one patient showed mitochondrial antibodies which corresponds to the finding in the control material

Even though there are no morphological or biochemical differences between the two groups, there are at the follow-up significantly more cases which develop cirrhosis in the group with abnormal biliary epithelium There can thus be no doubt about abnormal biliary epithelium being a bad prognostic sign, and this is further accentuated by the fact, that abnormal biliary epithelium was demonstrated also in the two cases of cirrhosis which developed in the group without abnormal biliary epithelium That these two patients were registered in this group is possibly due to a sampling error, as it is quite possible, that abnormal biliary epithelium was present already at the time of the first biopsy, but was just not represented in the biopsy, even though all the material was cut as serial section

It is difficult to express any opinion as to the reason for the group with abnormal biliary epithelium having a prognosis so much poorer than the group without It is difficult to imagine, that a possible obstructive effect of the abnormal epithelium should be the cause If this was the case, one would expect that the obstruction of the biliary tract, possible with obliteration and sclerosing fibrosis, would play an important part both morphologically, clinically and immunologically, and that the process would develop into a primary biliary cirrhosis This is not the case

The absence of Au antigenemia and the higher frequency of some autoantibodies in the group with abnormal bile duct epithelium suggest that the cases from the two groups may be etiologically distinct with more bile ductogenic affinity of the probably viral agent causing hepatitis in group 1 or a more pronounced effect of auto immunological factors contributing to the pathogenesis causing a graver prognosis

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DISAPPEARANCE OF CIRCULATING TUMOUR CELLS IN MICE TREATED WITH HEPARIN, COUMARIN AND EACA

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In a syngeneic tumour host system the disappearance of circulating tumour cells was studied in mice treated with heparin a coumarin anticoagulant and an antifibrinolytic The cells were injected intravenously in two doses and blood was drawn from the right heart at intervals after the cell transfusion and inoculated intraperitoneally into new recipients Only minor changes in the disappearance rate of tumour cells from the blood were obtained by anticoagulant and antifibrinolytic treatment Thus thrombus formation if present cannot play but a minor role for the retention of tumour cells in organs in this system The coumarin anticoagulant delayed somewhat the removal from the blood of 2×10^6 injected tumour cells This was in contrast to heparin which if anything seemed to hasten the removal of the cells from the blood

Experiments by cytological radiochemical and bioassay methods have shown that tumour cells can pass through capillary beds and remain in the circulation for some time (*Griffiths & Salsbury* 1965)

Thrombi found around tumour cells captured in different organs have been regarded as necessary for or at least as promoting metastasis formation (*Clifton & Agostino* 1965 *Ryan et al* 1968) We have not however, found anticoagulants to inhibit the development of metastases from syngeneic tumours (*Boeryd* 1966b *Hagmar* 1970) But in some experiments the metastasis pattern has been changed by anticoagulants These findings have been attributed to an altered distribution of tumour cells perhaps related to an altered circulation time

The aim of the present investigation was to study whether heparin a coumarin anticoagulant (phenprocoumon) and an antifibrinolytic (EACA) will affect the circulation time of syngeneic tumour cells These drugs have previously been used extensively in experiments related to the influence of changed clottability on metastasis formation

MATERIAL AND METHODS

The experiments were performed on inbred CBA mice using the MCA induced MCG1 SS (*Mellgren et al* 1966) The tumour cell suspensions were produced enzymatically using trypsin and DNase (*Boeryd et al* 1965) To obtain monodisperse suspensions and retain maximal viability the suspensions were resuspended in Parker 199 containing 10 per cent syngeneic mouse serum and kept at 0°C (*Norrby et al* 1966 *Knutson et al* 1971) The cells were counted in a haemacytometer and cell viability estimated by the Trypan blue exclusion test was over 85 per cent in the suspensions used

Two cell doses were tested 5×10^4 and 2×10^6 cells in 0 1 ml medium The cell suspensions were

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injected into a tail vein of the pretreated (see below) donor mice A mouse was taken successively from each group for the injections After the appropriate time interval (Tables 1 and 2) 0,7 ml of blood were drawn from the right side of the heart into a syringe containing 0 1 ml of 0 1 M sodium citrate This was performed after opening of the thoracic cavity along the midline During the aspiration of blood the animals were kept under ether narcosis The citrated blood from a donor animal was immediately injected intraperitoneally into an untreated recipient mouse These mice were observed until spontaneous death occured when they were autopsied Death was invariably due to extensive intraperitoneal tumour growth with ascites production

The donor mice were pretreated as follows

Controls 0 02 ml saline subcutaneously 1 hour before and 7 and 15 hours after the cell injection

Heparin 0 02 ml (1 mg) subcutaneously 1 hour before and 7 and 15 hours after the cell injection

Phenprocoumon (Marcoumar, generously supplied by F Hoffman–La Roche & Co, Basel, Switzerland) 0 02 ml (0 02 mg) intraperitoneally 27 hours and 1 hour before the cell injection 0 02 ml saline was given 7 and 15 hours after the cell injection

EACA (epsilon aminocaproic acid, generously supplied by AB Kabi Stockholm Sweden) 30 per cent in a powdered diet fed *ad libitum* for 24 hours before the cell injection In addition these animals were given saline subcutaneously like the controls

The doses and time intervals for treatment were chosen on the basis of previous studies on the effect of the drugs on the coagulability and fibrinolytic activity of the blood (*Boeryd* 1965, *Hagmar* 1968)

The number of animals given each cell dose is shown in Tables 1 and 2

Differences in the incidences of tumour takes were tested with the Chi square test Differences with $p < 0 05$ were regarded as significant

RESULTS

With a cell dose of 5×10^4, viable cells were removed from the blood within 6 hours after injection (Table 1) In fact, after half an hour only about 50 per cent of the animals were positive, and by 2 hours after injection most animals had no transplantable cells There were no significant differences between any of the groups

With the larger dose of 2×10^6 cells, viable cells could be found in some animals until 12 hours after injection (Table 2) This was true in all but the heparin-treated group, where no cells were found at that time Until 2 hours after injection all animals were positive in all groups After 6 hours about half of the animals had circulating cells, except in the phenprocoumon treated group Blood from the animals in this group gave a significantly higher number of takes at that time than any of the other groups

TABLE 1 *Incidence of Intraperitoneal Tumour* Takes after Injection of 0 7 ml of Heart Blood from Animals Injected with $5 \quad 10^4$ MCG1 SS Cells Intravenously Blood Drawn at Time Intervals Indicated in the Table

	0 5 hrs	2 hrs	6 hrs
Controls	3/8	2/8	0/4
Heparin	4/8	0/8	0/4
Phenprocoumon	4/9	2/9	0/4
EACA	5/8	3/8	0/4

DISCUSSION

Cytological methods for the detection of tumour cells in blood involve the difficulties of identifying the cells and assessing their viability The transplantation of blood to fresh syngeneic animals and observation of resulting tumour growth allows selective detection of circulating, viable cells

The sarcoma MCG1-SS is transplantable intraperitoneally with about 10 cells (*Boeryd et al* 1971) Thus using this tumour and method of transplantation, even a very

TABLE 2 Incidence of Intraperitoneal Tumour Takes after Injection of 0 7 ml of Heart Blood from Animals Injected with $2 \quad 10^6$ MCG1-SS Cells intravenously Blood Drawn at Time Intervals Indicated in the Table

	0 5 hrs	2 hrs	6 hrs	12 hrs	24 hrs
Controls	3/3	3/3	7/14	3/9	0/3
Heparin	3/3	3/3	6/13	0/8	0/3
Phenprocoumon	3/3	3/3	12/14	2/9	0/3
EACA	3/3	3/3	5/14	4/9	0/3

small number of viable cells in the blood will be detected In contrast another tumour subject to metastasis studies melanoma B 16 in C57 BL/6J mice, is transplantable intra peritoneally in a dose of about 10^3 cells With this tumour it has not been pos sible to recover viable cells even within the first hour after intravenous injection of 10^5 cells (unpublished results)

The presence of MCG1 SS cells in the blood for some hours after injection proves the passage of these cells through vascular beds probably repeated passages through se veral organs Similar results have been ob tained in allogeneic systems (*Agostino et al* 1961 *Koike* 1964) The MCG1 SS cells were present in the blood for a longer time after injection of 2×10^6 cells than after injection of 5×10^4 cells This indicates that the me chanisms for tumour cell removal from the blood although as yet incompletely known are cell dose dependent

The anticoagulant and antifibrinolytic the rapy given did not dramatically affect the disappearance rate of tumour cells from the blood minimizing the possible importance of thrombi for this process Six hours after in jection of the larger cell dose (2×10^6 cells) coumarin treatment kept tumour cells circul ating in a significantly greater number of animals however This difference which had disappeared within 12 hours may indicate that fibrin thrombi play a certain role in the elimination of tumour cells from the circul ating blood Tumour cells can be enmeshed in fibrin intravenously (*Wood* 1958 1964) Whether this fibrin promotes or prevents the penetration by cells of the vascular wall is a matter of controversy In allogeneic systems the presence of thrombi has been assumed to promote cell penetration and thereby meta stasis development (*Wood et al* 1961 *Cliffton* & *Agostino* 1965) However phenpro coumon increased the total crop of intrave nously induced metastases from two syngen eic tumours MCG1 SS and melanoma B 16 (*Hagmar* & *Boeryd* 1969) With MCG1 SS the increase occured in extrapulmonary or gans One explanation of this finding could be a delayed removal of cells from the blood during some critical hours offering the cells more chances for capture in various organs.

It is surprising that heparin did not influ ence the tumour cell removal in the same way as phenprocoumon If anything heparin seemed to hasten the removal of MCG1 SS cells from the blood Thus the tentative ex planation of heparin s effect on the distribut ion of experimental metastases (*Boeryd* 1966a, *Hagmar* & *Boeryd* 1969), i e a pro longed circulation time, could not be sub stantiated *Agostino et al* (1961) also found a marked decrease in the transplantability of Walker 256 carcinoma cells from the blood of heparin treated rats Conversely heparin pro longed the circulation time of tumour cells in other systems, tested by bioassay (*Koike* 1964) or cytological techniques (*Stevenson* & *von Haam* 1965)

Since herapin may alter the tumour cell surface (*Nordling* 1967, *Ambrose* 1967, *Hagmar* & *Norrby* 1970) an altered relationship to the vascular endothelium could follow This change would imply a higher electrone gativity of the cell surfaces It is difficult to imagine how this change could lead to a pos sibly quicker removal of the cells from the blood An explanation of this unexpected finding might be that heparin hinders the lodgement and growth of cells intraperitone ally (*Goldie et al* 1961) and thus affects the method of cell detection A devitalizing effect on the cells by heparin seems unlikely in view of cytotoxicity studies performed with MCG1 SS cells (*Hagmar* 1969 *Hagmar* & *Norrby* 1970)

EACA increased the amount of pulmonary metastases from intravenously injected tu mour cells in repeated studies with three dif ferent tumours (*Boeryd* 1965 1966a *Boeryd* & *Rudenstam* 1967 *Boeryd et al* 1971) This was interpreted in terms of an increased re tention of tumour cells in the lungs by inhi bited fibrinolysis and/or haemodynamic chan ges (*Boeryd et al* 1971) If so this was not in the case of MCG1 SS reflected in a more rapid disappearance of tumour cells from the blood However the method used here only

reflects the total retention of cells in organs in relation to blood The possibility of an alteration in the distribution of cells between different organs remains to be tested For such more detailed analysis, the organ transplantation test of *Donelli et al* (1969) or, perhaps more conveniently, radioassay procedures (*Fidler* 1970) have to be used

This investigation was supported by grants from the *Swedish Cancer Society*

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INFLUENCE OF SUPERIOR CERVICAL SYMPATHECTOMY ON TRAUMATICALLY INDUCED BRAIN OEDEMA

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Traumatic brain oedema was induced in rabbits by trephine craniotomy which causes circumscribed thermal injury to underlying structures The amount of oedema was estimated as the per cent water content in brain as determined after heat combustion The water content was thus measured in the brain of non sympathectomized animals and after bilateral superior cervical sympathectomy, with or without additional trephine traumatization Changes in brain water simply reflecting altered cerebral blood volume after sympathectomy could be corrected for Sympathectomy 32 hours before water determination caused about 50 per cent reduction in the amount of traumatic brain oedema At 5 days the degree of oedema was of the same magnitude as in the non sympathectomized animals subjected only to trephination Fifteen days after sympathectomy the amount extended 50 per cent above that in the trephinized non sympathectomized animals *It is suggested that the cranial sympathetics can modify the extent of a traumatic brain oedema through its action on the cerebrovascular system*

The pial arteries have been shown to receive a rich sympathetic nerve supply emanating from the superior cervical ganglia (*Nielsen & Owman* 1967 *Spoendlin & Lichtensteiger* 1967, *Donath* 1968 *Falck et al* 1968 *Ohgushi* 1968 *Kajikawa* 1968, 1969) Bilateral removal of these ganglia results within 2 days in a disappearance of the neurotransmitter noradrenaline (NA) from the pial vascular sympathetic nerves (*Nielsen & Owman* 1967, *Edvinsson et al* 1972b) The leakage and subsequent disappearance of NA from the degenerating nerve terminals, and the progressively increasing denervation supersensitivity of the vascular smooth muscles to circulating catecholamines have been shown to alter the cerebral blood volume (*Edvinsson et al* 1971d) and the intracranial pressure as measured via a cannula implanted into the left lateral ventricle of the brain in conscious rabbits (*Owman & West* 1970 *Edvinsson et al* 1971a) The denervation changes the carbonic anhydrase activity in the choroid plexus indicating a possible sympathetic influence also on the formation of the cerebrospinal fluid (*Edvinsson et al* 1972a)

The cannula used in the pressure recordings was found to affect the *intracranial* pressure by producing a local traumatic brain oedema (*Edvinsson et al* 1971a) which was taken into account when correcting the pressure recordings obtained after interference with the cranial sympathetic nerves (*Edvinsson et al* 1971b c and 1972c) The present investigation was undertaken to test whether

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sympathectomy apart from influencing the intracranial pressure *per se*, also affected the degree of brain oedema produced by trephine craniotomy (*Edvinsson & West* 1972)

MATERIAL AND METHODS

The material comprises 35 rabbits of either sex weighing 2–3 kg. The animals were fed with standard pellet food (SAN bolagen, Sweden), turnips, carrots and tap water *ad lib* during the entire experiment. The animals were with respect to age distributed among the various groups so that the mean body weight did not differ significantly between the separate groups.

Brain oedema, the phenomenon of abnormal increase and accumulation of fluid extracellularly and/or intracellularly in the brain tissue, was produced by placing 3 pairs of burr holes (Group Bu) bilaterally to the midline of the skull with a trephine burr (3.2 mm in diameter). The fronto-parieto-occipital region of the skull convexity was exposed in 5 animals under local anaesthesia (3 ml of 2 per cent lidocain, Xylocain, Astra, Sweden) through a midline skin incision as previously described (*Edvinsson & West* 1972). When the trephination is performed without simultaneous irrigation of the skull bone with physiological saline it is known to cause a local brain oedema of reproducible size in the adjacent brain cortex (*Edvinsson & West* 1972). The burr holes were then tightly sealed with surgical wax and the skin incision was closed with sutures. The animals were killed by intravenous air injection 26 hours after placing the burr holes. The telencephalic brain including the caudate nucleus was dissected out, immediately weighed, dried for 24 hours at 120° C in an electrical oven and re-weighed. The water content was expressed as per cent of the brain tissue weight before drying. Five untreated animals served as controls (Group Co).

Under light dinitrous oxide-halothane (Fluothane, I.C.I.) inhalation anaesthesia the superior cervical sympathetic ganglia were removed bilaterally in 15 animals (Group Sy) through a midline skin incision in the neck as earlier described (*Edvinsson et al.* 1971b). After 32 hours (5 animals), 5 days (5 animals) and 15 days (5 animals) the telencephalic brain was removed and dried at 120° C as described above.

In another series of experiments ganglionectomy was combined with trephination (Group SyBu). Thus 15 animals were subjected to bilateral superior cervical ganglionectomy 6 hours (5 animals), 4 days (5 animals) and 14 days (5 animals) before burr holes were placed in the manner earlier described. The animals were killed 26 hours later and the telencephalic brain was removed and dried.

On the basis of the figures for water contents in the 4 groups of animals (Group Co, Bu, Sy and SyBu) the influence of the various procedures on the fluid contents could be calculated as follows:

(a) Values of Group Sy minus values of Group Co give the change in brain water due to ganglionectomy, probably dependent upon changes in the volume of the vascular bed.

(b) Values of Group SyBu minus the change in brain water due to ganglionectomy (Sy minus Co) specifie the change in water content due to trephination of the ganglionectomized animals.

(c) The effect of ganglionectomy on the traumatically induced brain oedema was obtained by subtracting the values of Group Bu from the change in water content due to trephination of the ganglionectomized animals (SyBu minus [Sy minus Co]). The values obtained will be referred to as the net oedema.

In order to check the completeness of sympathetic denervation of the pial arteries the main pial arteries in the pial membrane at the base of the brain were spred on microscope slides. After drying for 1 hour in a desiccator over phosphorous pentoxide the preparations were exposed to formaldehyde gas at 80° C during 1 hour for the fluorescence histochemical demonstration of the NA transmitter in the vascular sympathetic nerves (see *Falck & Owman* 1965). A green fluorescence in the perivascular nerves was indicative of the presence of NA in the nerves.

RESULTS

The telencephalic brain water content (Mean ± S.E.M.) in the 5 untreated control animals (Group Co) was 79.91 ± 0.08 per cent. The 5 animals subjected to burring of the skull bone (Group Bu) had a mean brain water content of 81.27 ± 0.21 per cent. The water content in brain of those animals, that had been subjected to burring had thus increased with 1.36 per cent compared with controls (Group Bu minus Group Co). This value represents the traumatic brain oedema.

The telencephalic brain water content at the 3 time intervals after sympathectomy (Group Sy) was 80.30 ± 0.11, 79.94 ± 0.11, and 79.38 ± 0.13 per cent respectively. The change in the brain water content due to the bilateral ganglionectomy was obtained by subtracting the telencephalic brain water content in the untreated control group from

these values (i e Group Sy minus Group Co) The resulting values were +0 39 + 0 03 and —0 53 The change in brain water content specifically due to the trephination trauma was obtained by subtracting these 3 values from the corresponding time related values after combined sympathectomy and trephination (Group SyBu) 80 96 ± 0 38 81 35 ± 0 30, and 81 40 ± 0 18 per cent The values thus calculated for the 3 time intervals were 80 57, 81 32 and 81 93 per cent By subtracting the mean value obtained in the trephinized animals (Group Bu) from these latter values the specific effect (net oedema) of superior cervical sympathetic denervation on induced traumatic brain oedema was obtained The following values for net oedema

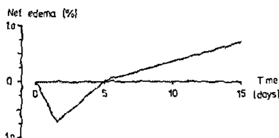


Fig 1 Net oedema (for definition see text) in brain at different time periods after bilateral excision of the superior cervical sympathetic ganglia The oedema was the result of the thermal brain injury caused by trephine craniotomy 26 hours before the animal was killed at each time interval

could thus be calculated —0 70 per cent 0 05 per cent and 0 66 per cent (Fig 1) It is evident from Fig 1 that ganglionectomy modifies the brain oedema produced by the trephine trauma so that it is reduced by about 50 per cent 1 day after sympathectomy and increased by the same amount 15 days after the operation

Fluorescence microscopy revealed that the fluorescence intensity (and thus the neuronal NA content) in the perivascular sympathetic nerve fibres around the pial vessels appeared to be unaltered in the non ganglionectomized animals with traumatic brain oedema as compared with the unoperated control group In the groups of animals subjected to ganglionectomy 32 hours before killing the fluorescence intensity in the sympathetic vascular nerves was very faint, regardless of whether the animals were trephined or not This indicated a pronounced decrease in the NA content of the sympathetic vascular nerves at this postoperative stage The groups of animals ganglionectomized 5 and 15 days before killing were totally devoid of microscopically visible NA fluorescence in their vascular sympathetic nerves

DISCUSSION

There is recent experimental evidence that the cranial sympathetics influences the intracranial pressure (*Owman & West 1970*) The findings obtained by surgical interference with the superior cervical sympathetic ganglia (pre and postganglionic denervation) have indicated that the influence is exerted mainly via vasoconstrictor adrenergic nerves (*Edvinsson et al* 1971d and 1972d *Nielsen & Owmann* 1971) acting on the cerebral blood volume (*Edvinsson et al* 1971b c and 1972c) The results included correction for those additional alterations in the intracranial pressure caused by the local traumatic brain oedema around the implanted cannula (*Edvinsson et al 1971a*) Also when burr holes are placed in the skull bone by a trephine burr a cortical and subcortical oedema develops in the underlying brain structures from the thermal injury (*Edvinsson & West 1972*) Microscopically this oedema manifests itself through an accumulation of fluid within glial elements in the gray matter and through collection of oedema fluid in the extracellular space of the white matter The oedematous changes in the brain were most pronounced during the second day after induction and the oedema then subsides within 2 weeks The development of brain oedema is associated with an increase in the intracranial pressure (*Edvinsson et al 1971a*)

It appears from the present report that the surgical interference with the superior cervical ganglia modifies the amount of traumatic brain oedema and furthermore that the

modification is time related with regard to the operative procedure Thus when ever intracranial pressure after sympathetic denervation from even a small but still traumatizing cannula placed in the brain via a burr hole is to be recorded not only the methodological error has to be taken into consideration—the traumatic brain oedema—but also any effect the denervation may have in modifying the extent of this oedema. Considering the net oedema curve and the time corresponding pressure sequences previously reported (*Edvinsson et al* 1971b c and 1972c) it is evident that an exceptionally low degree of oedema is included in the relatively low intracranial pressure seen shortly after sympathectomy, and that the normal or even subnormal pressure after 2 weeks includes an exceptionally high amount of traumatic oedema

The amount of oedema has been estimated on the basis of calculated water content in the brain after combustion Corrections have been made for the contribution of the volume of circulating blood in the vascular bed Otherwise the influence of sympathectomy on the traumatically induced oedema would have been highly masked by the blood volume variations due to sympathectomy Histological evidence indicates that the oedema is combined intra and extracellular (*Edvinsson et al* 1971a *Edvinsson* & *West* 1972) It was mentioned that sympathectomy significantly affects cerebral blood volume (*Edvinsson et al* 1971d and 1972d) The present results have shown that interference with the cranial sympathetics also changes the brain water content Since however sympathectomy *per se* does not induce brain oedema (*Shackelford* & *Hegedus* 1961) it was assumed that these changes in brain water at least mainly reflect alterations in cerebral blood volume On the other hand it can be expected that in the presence of a traumatic oedema the amount of water in the oedematous region is supplied from the altering vascular bed so that a vasoconstriction (reduced cerebral blood volume) favours an increment in the volume of the oedema and that vasodilation (increased cerebral blood volume) promotes an efflux of oedema fluid

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search organization sponsored by the *Swedish Medical Research Council* (projects No B71-14X-712-06A and B71 14X 56-07A)

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PREDETERMINED SEQUENTIAL CHROMOSOME CHANGES IN SERIAL TRANSPLANTATION OF ROUS RAT SARCOMAS

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The early chromosomal progression *in vivo* was studied in 5–6 parallel series of passages of 2 primary Rous rat sarcomas (RSV-SR) Both sarcomas had a normal diploid stemline, no sideline and no deviating cells During transplantation all series of both tumours showed a heteroploid transformation There were indications that the initial change in the hetero ploid evolution was predetermined with a specificity comprising not only the direction of the heteroploid pathway (hyperdiploidy), but also the karyotype Thus, among all series of passages derived from the same primary sarcoma the earliest deviating cells had exactly the same karyotype In both tumours the early heteroploid evolution displayed the same non random and sequential characteristics gains of chromosome types t st_3 and st_5 respectively In the course of the heteroploid evolution the normal diploid cells decreased by a non linear process with a slow initiation and a rapid fall The chromosomal progression from a normal diploid stemline to a heteroploid stemline was related to a histological dedifferentiation

The chromosomal findings in 63 primary and 20 metastatic Rous rat sarcomas have recent ly been reported (*Mitelman* & *Mark* 1970, *Mitelman* 1971 1972) The results were com patible with the conclusion that all the primary tumours had started out with nor mal diploid cells When a heteroploid trans formation had been initiated certain evolu tionary pathways seemed to be preferred, hyperdiploidy was the most important, fol lowed by pseudodiploidy, while hypodiploidy and polyploidy were stemline categories rarely observed The karyotypic changes in the heteroploid evolution were non random and had similar characteristics in both the primary and the metastatic tumours In ad dition, there were many indications that the sequence of these changes was predetermined

The aim of the present investigation was to test this hypothesis of sequential karyotypic events, to elucidate whether additional steps could be discovered and to find out to what extent the karyotypic changes in different series of passages were predetermined Two primary Rous rat sarcomas with a normal diploid stemline and lacking deviating cells were selected From each tumour 5 and 6 parallel series of passages, respectively, were established, in each series the tumour was transplanted to approximately 20 succeeding generations of rats Below, the chromosomal

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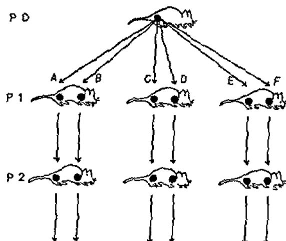


Fig 1 Diagram of the transplantations

and the histological findings in these 11 series of passages are presented

MATERIAL AND METHODS

The two primary sarcomas selected for the present experiments were the female tumour No 2 and the male tumour No 4 of a previous study (*Mitelman* 1971) Both were induced with a cell free suspension of Rous chicken sarcoma virus strain Schmidt Ruppin (RSV–SR) For details see *Mitelman* (1971) The rats used for the induction of the primary sarcomas and for the transplantations belonged to the same inbred Wistar/Furth (W/Fu) strain propagated at our institute by strict brother sister mating (continuous single line) New born rats were used for the first 3–4 *transfer* generations, while only adult animals were used for the subsequent passages

The transplantations were carried out by inoculating 0 2 ml of a finely minced 1 5 suspension of the tumour in Hank s solution in the right hind leg and the left fore leg of 3 new hosts as illustrated in Fig 1 The new hosts were always of the same sex as the rat in which the primary sarcoma was induced Sarcoma No 2 was followed in 6 series (A F) (Fig 1) during 16–20 passages sarcoma No 4 was followed in 5 series (A E series F was omitted due to infection in the second passage) during 19 23 passages

The chromosomes were studied intermittently in each series usually every other passage In all 121 tumours were analysed chromosomally All tumours were studied in fixations directly from the tumour the method was described in detail earlier (*Mitelman & Mark* 1970) At least 50 cells were counted from each tumour 10 cells or more were karyotyped from each stemline and 5 cells or more from each sideline Karyotype analyses were made by photography

All of the tumours studied chromosomally were also examined histopathologically The pieces of the tumour chosen for histopathological examination were fixed in 10 per cent formalin and routinely stained with haematoxylin eosin and van Gieson All tumours were recognized as fibrosarcomas with varying degree of maturity Three histological classes could be distinguished (F) highly differentiated fibrosarcoma (Sp) spindle cell sarcoma, and (A) anaplastic round cell sarcoma with giant cells The criteria used for this classification were the same as those used by *Mitelman* (1971 1972), the classification was performed without knowledge of the chromosomal data

TERMINOLOGY

The system of classification of the rat chromosomes (Fig 2) and the terminology of chromosome numbers are the same as those in *Mitelman* (1971) As in that report S is a symbol for the stemline *i e* the most frequent karyotype of the tumour cell population and s (s_1 s etc) a symbol for the sideline(s), *i e* other karytypes of the tumour cell population present in a frequency of 10 per cent or more

Nomenclature for centromeric position follows the recommendation of *Levan et al* (1964) This nomenclature is also used for marker chromosomes with Mk either as a suffix or abbreviation

RESULTS

Primary Sarcoma No 2

Primary sarcoma No 2 was a female tumour with a normal diploid stemline and no sideline Of this tumour 100 cells were counted, all metaphases had the *normal diploid* chromosome number Twenty cells were analysed all of these had a normal *diploid* karyotype (Fig 2 a) This tumour was studied in 6 parallel series (A–F) *during* 16–20 *in vivo* passages The chromosomal and histological findings are summarized *in* Table 1

In all of the 6 series of passages the first *step in the modification* of the normal diploid stemline was the appearance in *P2–P7* of isolated 43–chromosome cells In all series *these* variant cells had the same karyotype *differing from the normal* by the loss of 1 st and the gain of 1 st and *1 b g submedian*

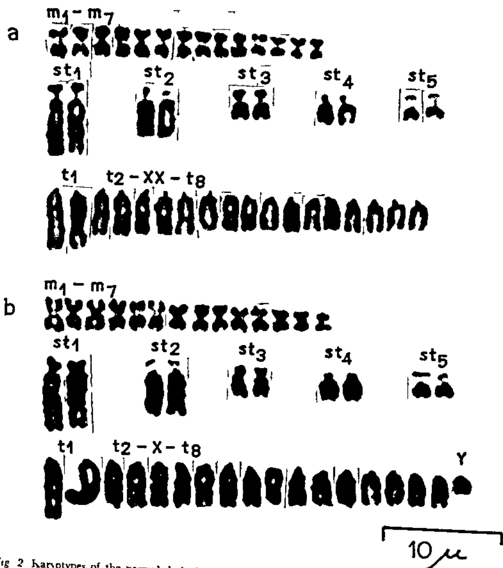


Fig 2 Karyotypes of the normal diploid stemlines of the two primary Rous rat sarcomas a Primary sarcoma No 2 b Primary sarcoma No 4

marker One of these cells is illustrated in Fig 3 The sm marker had an arm ratio of 2 2 In all cases the length of the long arm corresponded exactly to that of the long arm of an st chromosome the short arm was slightly shorter than the long arm of an st_3 chromosome and its length did not correspond to any of the chromosome arms of the rat complement Since 1 st was missing it was reasonable to assume that this chromosome had contributed to the long arm of the marker, the origin of the short arm could not be established (cf discussion)

In all series this 43 chromosome variant cell increased in prominence and was seen 2–4 passages later as stemline in series B, D and F, and as sideline in series A C and E In series A the sideline developed rapidly and replaced the normal diploid stemline in the following passage whereas modifications of the sidelines preceded the S shift in series C and E

TABLE 1 *The Chromosomal and Histological Progress*

Series	Pass No	Growth period, days	Stemline 2n %	Karyotype as related to normal diploid karyotype
A	1– 3	48– 92	42 (100)	≈
	5	118	42 (98)	≈
	7	157	42 (65)	≈
	8	168	43 (40)	+1Mk +1st$_3$ —1st
	10	193	45 (66)	+1Mk +1st$_3$ —1st +1st$_5$ +1t
	13	222	45 (87)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$ +1t
	16	255	45 (100)	+1Mk +1st$_3$ —1st +1st$_5$ +1t
B	1	48	42 (100)	–
	3	92	42 (96)	≈
	5	118	43 (63)	+1Mk +1st$_3$ —1st.
	7	157	43 (38)	+1Mk +1st$_3$ —1st
	9	179	43 (84)	+1Mk +1st$_3$ —1st —1st$_5$ +1m
	12	209	43 (60)	+1Mk +1st$_3$ —1st —1st$_5$ +1m
	14	234	43 (38)	+1Mk +1st$_3$ —1st. —1st$_5$ +1m
	16	255	43 (46)	+1Mk +1st$_3$ —1st —1st$_6$ +1m
	19 20	290–303	45 (76 88)	+4Mk +1st$_3$ —2st$_2$ +2st$_5$ —1st$_4$ —1t$_1$
C	1	62	42 (100)	–
	2	84	42 (98)	–
	4	101	42 (76)	≈
	6	124	42 (80)	–
	8	170	42 (80)	–
	10	214	42 (82)	–
	11	225	42 (72)	–
	12	240	42 (76)	–
	14	266	46 (68)	+1Mk +1st$_3$ —1st$_2$ +1st +1t +1t$_1$
	16 18	293 316	46 (84–75)	+1Mk +1st$_3$ —1st$_2$ +1st +1t +1t$_1$
D	1– 4	62–101	42 (100)	–
	6	124	42 (94)	≈
	8	170	43 (82)	+1Mk +1st$_3$ —1st$_2$
	10	214	43 (52)	+1Mk +1st$_3$ —1st
	13	254	44 (32)	+2Mk +1st$_3$ —1st$_2$ +1st —1m
	15	281	45 (58)	+2Mk +1st$_3$ —1st$_4$ +1st$_6$
	16	293	45 (82)	+2Mk +1st$_3$ —1st +1st$_5$
	18	316	45 (72)	+2Mk +1st —1st +1st$_3$
E	1	56	42 (100)	
	3 5	84 111	42 (98–94)	–
	7	147	42 (36)	
	8	163	42 (20)	≈
	10	202	44 (40)	+1Mk +1st$_3$ —1st 1st$_5$
	13	249	44 (31)	+1Mk +1st$_3$ —1st 1st
	15	277	45 (26)	+1Mk +1st$_3$ 1st +1st +1m
	18	320	90 (57)	+2Mk +2st$_3$ —2st$_2$ + st +2m*
F	1– 5	56 111	42 (100)	–
	7 10	147 202	42 (90 88)	≈
	11	221	43 (44)	+1Mk +1st$_3$ —1st.
	13	249	43 (59)	+1Mk +1st$_3$ —1st
	15	277	44 (81)	+1Mk +1st$_3$ —1st$_2$ +1st +1t$_1$ —1t
	20	346	42 (54)	+1st$_3$ 1st +1st$_5$ 1 2t

* In relation to normal tetraploid karyotype

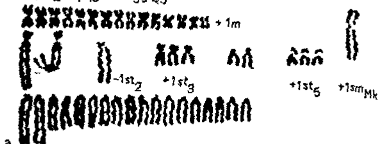
Sidelines			
s_1 2n %	Karyotype as related to normal diploid karyotype	s_2 2n %	Karyotype as related to normal diploid karyotype
—		—	
—		—	
43 (26)	+1Mk +1st$_3$ —1st.	—	
44 (35)	+1Mk +1st$_3$ —1st. +1st$_5$	45 (20)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$ +1t
44 (17)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$	—	
—		—	
—		—	
—		—	
—		—	
42 (23)	=	—	
42 (30)	+1Mk +1st$_3$ —1st. —1m	43 (24)	+1Mk +1st$_3$ —1st$_2$ —1st$_5$ +1m
—		—	
44 (12)	+4Mk +1st$_3$ —2st$_2$ +1st$_5$ —1st$_4$ —1t$_1$	45 (10)	+4Mk +1st$_3$ —2st. +2st$_5$ —1st$_4$ —1t$_1$
44 (24)	+4Mk +1st$_3$ —2st$_2$ +1st$_5$ —1st$_4$ —1t$_1$	45 (16)	+4Mk +1st$_3$ —2st$_2$ +2st$_5$ —1st$_4$ —1t$_1$
45 28)	+4Mk +1st$_3$ —2st$_2$ +2st. —1st$_4$ —1t$_1$	—	
—		—	
—		—	
—		—	
43 (10)	+1Mk +1st$_3$ —1st$_2$	—	
43 (10)	+1Mk +1st$_3$ —1st$_2$	—	
43 (14)	+1Mk +1st$_3$ —1st.	—	
43 (10)	+1Mk +1st$_3$ —1st.	—	
43 (11)	+1Mk +1st$_3$ —1st.	45 (10)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$ +1t
45 (12)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$ +1t	43 (10)	+1Mk +1st$_3$ —1st.
45 (15)	+1Mk +1st$_3$ —1st. +1st$_5$ +1t	—	
—		—	
—		—	
—		—	
42 (11)	=	—	
44 (35)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$	—	
45 26)	+2Mk +1st$_3$ —1st. +1st$_5$	43 (18)	+1Mk +1st$_3$ —1st$_2$ +1st$_5$ —1m
44 (18)	+2Mk +1st$_3$ —1st. +1st$_5$ —1m	—	
—		—	
44 (16)	+2Mk +1st$_3$ —1st$_2$ +1st$_5$ —1m	—	
		—	
—		—	
13 (19)	+1Mk +1st$_4$ 1st	44 (17)	+1Mk +1st$_3$ —1st +1st$_5$
43 18)	+1Mk +1st$_1$ —1st.	44 (15)	+1Mk +1st$_3$ —1st$_2$ +1st.
45 (32)	+1Mk +1st$_1$ 1st$_2$ +1st. +1m	—	
45 (30)	+1Mk +1st$_1$ —1st. +1st$_5$ +1m	—	
40 15)	+2Mk +2st$_1$ 2st. +2st$_5$ +2m*	—	
		—	
		—	
		—	
42 26	=	—	
44 (14)	+1Mk +1st$_1$ 1st$_2$ +1st. +1t$_1$ —1t	43 (11)	+1st$_3$ —1st +1st. +1t$_1$ —1t
43 (12)	+1st$_3$ —1st$_2$ +1st$_5$ +1t$_1$ —1t	—	
43 (22	+1st$_3$ —1st +1st$_5$ +1t$_1$ —1t	—	

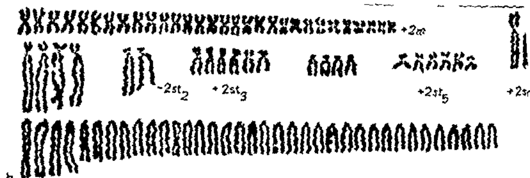
Series	Pass No	Growth period, days	Stemline 2n %	Karyotype as related to normal diploid karyotype
A	1– 2	74– 86	42 (100)	=
	3– 9	101–168	42 (94–86)	=
	11	197	42 (60)	=
	13	224	42 (56)	=
	14	237	42 (44)	=
	15	251	45 (34)	+ 2t + $1st_5$
	17	280	46 (74)	+ 2t + $1st_5$ + 1t
	19	307	46 (86)	+ 2t + $1st_5$ + 1t
B	1	74	42 (100)	=
	2– 3	86–101	42 (94–92)	=
	5	121	42 (82)	=
	7	140	42 (80)	=
	9	168	42 (80)	=
	11	197	42 (80)	=
	12	212	42 (78)	=
	13	224	46 (54)	+ 2t + $1st_3$ + $1st_5$
	14	237	46 (58)	+ 2t + $1st_3$ + $1st_5$
	17	280	46 (43)	+ 2t + $1st_3$ + $1st_5$
	19	307	47 (48)	+ 2t + $1st_3$ + $1st_5$ + 1m
	20	319	47 (50)	+ 2t + $1st_3$ + $1st_5$ + 1m
C	1	68	42 (100)	=
	2– 5	82–115	42 (96–90)	=
	7	137	42 (80)	=
	9	166	42 (82)	–
	11	187	42 (78)	=
	13	218	42 (76)	=
	14	231	42 (42)	=
	16–20	257–310	88 (58–34)	+ 4t*
D	1	68	42 (100)	
	2	82	42 (92)	=
	4	106	42 (76)	=
	5	115	42 (78)	=
	8	149	42 (66)	=
	9	166	42 (76)	=
	11	187	44 (36)	+ 2t
	14	231	44 (30)	+ 2t
	15	242	45 (48)	+ 2t + $1st_3$
	17	272	46 (46)	+ 1t + $1st_3$ + $1st_5$ + 1Mk
	19	297	46 (68)	+ 1t + $1st_3$ + $1st_5$ + 1Mk
E	1	70	42 (100)	=
	2–14	81 240	42 (92–78)	=
	15	252	42 (82)	=
	16	266	44 (44)	+ 2t
	17	275	44 (34)	+ 2t
	19	297	44 (28)	+ 2t
	20	308	44 (32)	+ 1Mk + $1st_3$
	21	317	44 (38)	+ 1Mk + $1st_3$
	22	330	45 (42)	+ 1Mk + $1st_3$ + $1st_3$
	23	341	45 (36)	+ 1Mk + $1st_3$ + 1st

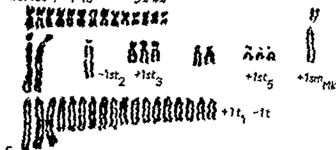
* In relation to normal tetraploid karyotype

in 5 Series of Passages of Primary Sarcoma No 4

Sidelines			
s_1 2n %	Karyotype as related to normal diploid karyotype	s_2 2n %	Karyotype as related to normal diploid karyotype
—		—	
—		—	
44 (20)	+2t	—	
44 (18)	+2t	45 (12)	+2t +1st$_5$
45 (20)	+2t +1st$_5$	46 (16)	+2t +1st$_5$ +1t
46 (24)	+2t +1st$_5$ +1t	44 (22)	+2t
45 (16)	+2t +1st$_5$	—	
—		—	
—		—	
—		—	
44 (16)	+2t	—	
44 (20)	+2t	—	
45 (16)	+2t +1st$_3$	—	
45 (10)	+2t +1st$_3$	46 (10)	+2t +1st$_3$ +1st$_5$
45 (10)	+2t +1st$_3$	46 (12)	+2t +1st$_3$ +1st
42 (22)	—	—	
47 (20)	+2t +1st$_3$ +1st$_5$ +1m	—	
47 (38)	+2t +1st$_3$ +1st$_5$ +1m	—	
46 (40)	+2t +1st$_3$ +1st	—	
46 (17)	+2t +1st$_3$ + 1st	—	
—		—	
—		—	
—		—	
44 (10)	+2t	—	
44 (12)	+2t	—	
44 (10)	+2t	—	
44 (34)	+2t	88 (11)	+4t*
—		—	
—		—	
—		—	
44 (14)	+2t	—	
44 (18)	+2t	—	
44 (24)	+2t	—	
44 (17)	+2t	—	
45 (30	+2t +1st,	—	
45 (26)	+2t +1st$_3$	45 (19)	+1t +1st$_3$ +1Mk
45 (38)	+1t +1st$_3$ +1Mk	44 (12)	+2t
45 (44)	+2t +1st$_3$	—	
45 ?6	+2t +1st$_3$	—	
—		—	
—		—	
44 (14)	+2t	—	
4? (20)			
44 (32)	+1Mk +1st$_3$	43 (20)	+1Mk
44 26)	+1Mk +1st$_3$	42 (18)	=
44 (24)	+2t	45 (16)	+1Mk +1st$_3$ +1st$_5$
45 (36)	+1Mk +1st$_3$ +1st$_5$	45 (15)	+1Mk +1st$_3$ +1st$_5$
44 (28)	+1Mk +1st$_3$	—	
44 (27)	+1Mk +1st$_3$	—	
		46 (12)	+1Mk +1st$_3$ +2st







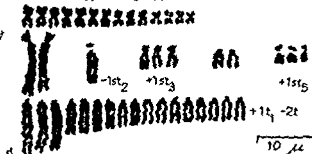


Fig. 5 a–d. Stemline karyotypes from series E–F of primary sarcoma No. 2

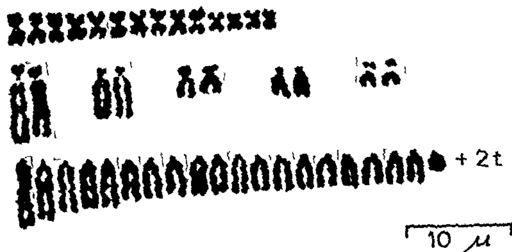


Fig 6 Karyotype of the 44-chromosome variant cell seen in all series of primary sarcoma No 4

minute marker had replaced 1 m chromosome (Fig 4 d) This minute marker was also of the t-type, but somewhat longer than in series B, the size corresponded approximately to that of the arms of one of the smallest m chromosomes Since 1 m was missing, the marker might have originated by a centromeric break in one of these chromosomes It should be mentioned that as the minute marker appeared in series D it was noticed that the short arm of the monosomic st_2 was definitely longer than normal (Fig 4 d) It is tempting to assume that this arm represents a translocation of the other part of the broken m chromosome In addition to these markers incorporated in the stem and sideline karyotypes numerous other marker types were occasionally seen in the variant cells of all series

In all series the initial progression was directed towards hyperdiploidy The S numbers observed were 43–46 Subsequently ...

a and b) and series F developed to pseudo diploidy (Fig 5 d) by rearrangements of a 43 chromosome sideline

Primary Sarcoma No 4

Primary sarcoma No 4 was a male tumour Out of 50 cells counted, all had 42 chromosomes and all the 10 cells analysed had the normal diploid karyotype (Fig 7 b) This tumour was studied in 5 series (A–E) during 19–23 succeeding passages The chromosomal and histological findings are summarized in Table 2

In all of the 5 series of passages the first step in the heteroploid evolution was the appearance of single hyperdiploid cells These variant cells, seen first in P2–P3, had 44 chromosomes and differed from the normal by a gain of 2 t chromosomes (Fig 6) After varying periods of time 2–13 passages, these karyotypes were seen as sidelines in all the different series In series D and E these sidelines developed into stemlines without other changes, while in series A, B and C further rearrangements occurred within the sidelines before the S shift

The immediate karyotypic step in the evolution of the 44-chromosome stem- or sidelines was a gain of 1 st_4 in series B, D and E and a gain of 1 st_5 in series A Series C showed a doubling of its 44 chromosome sideline and these doubling products replaced

21*

Series A P 19 S = 46

+ 1st$_5$

+ 3t

a

Series B P 20 S = 47

+ 1m

+ 1st$_3$

+ 1st$_5$

+ 2t

b

Series C P 20 S = 88

+ 4t

c

Series D P 19 S = 46

+ 1st$_3$

+ 1st$_5$ + 1t$_{Mk}$

+ 1t

d

Series E P 23 S = 45

+ 1st$_3$

+ 1st$_5$ + 1M$_{Mk}$

e

10 μ

Fig. 7 a–e Stemline karyotypes from series A–E of primary sarcoma ∖ 1

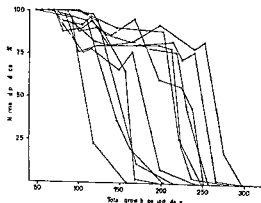


Fig 8 Relation between the frequency of normal diplo d cells in each series of passages and the total growth period of the tumours

the normal diploid stemline No further changes were seen in this series

The third step in series B D and E was the same in each case and identical to the second step in series A, *i e* 1 extra st In series A the third step was a gain of 1 t chromosome

Contrary to the previous tumour, other chromosomal changes were rare In fact the only additional numerical changes observed were a gain of 1 m chromosome in series B and losses of 1 and 2 t respectively, in series D and E Stemline karyotypes of the last passage studied of all series are shown in Fig 7 Structural rearrangements as measured by the presence of markers in the stemlines were seen in series D and E In P14 of series D 1 minute marker had replaced 1 t chromosome (Fig 7 d) The marker was of the t type, its size corresponded to that of the arms of one of the smallest m chromosomes The marker thus had approximately the same size as the minute marker seen in series D of the previous tumour Since in the present case no m, but 1 t was missing the marker might represent a deleted t chromosome In P16 of series E one big M marker had replaced 2 t chromosomes (Fig 7 e) The length of the arms of the marker corresponded to that of 1 of the t chromosomes of medium size Since 2 t chromosomes were actually missing a probable explanation for the origin of the marker is centric fusion of 2 mediumsized t chromosomes An observation worthy of mention is that in all series of passages of the present tumour strikingly few variant cells showed markers, in contrast to the previous tumour

As in the previous tumour, the progression in all series was initially directed towards hyperdiploidy Except for series C which showed the development of a hypertetraploid stemline (Fig 7 c) by a doubling of a previous 44 chromosome sideline, all the other series manifested hyperdiploid stemlines in the last passage studied The S numbers observed were 44–47

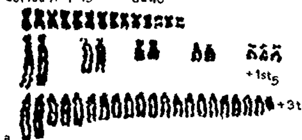
Frequency of Normal Diploid Cells and Growth Period of the Tumours

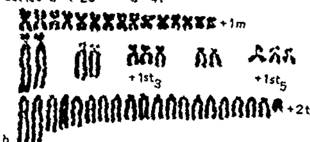
Fig 8 shows the frequency of the normal diploid cells in relation to the total growth period of each of the 11 series of passages As expected, the frequency decreases with increasing tumour age It is interesting however, that this process is non linear, characterized by a slow initiation, a rapid fall and a tendency to a slow end

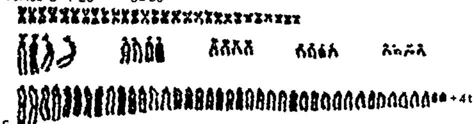
Further, it was found that the time from the beginning of the steep part of the curve to its end was quite constant in all series, *i e* 80–90 days This would indicate that the total period of time for the heteroploid population shift to be concluded is determined by the duration of the initiation period It should be mentioned that the slow initiation period encountered in all series might have been influenced by the fact that newborn rats were used for the first 3–4 transplant generations However since only adult animals were used from passages 4 5 onwards this could hardly have changed the character of the S curve

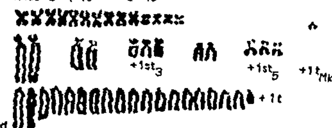
Chromosomes and Histopathological of the Tumours

Both primary sarcomas were [illegible] ferentiated fibrosarcomas The [illegible] types of the transplanted tumours in Tables 1 and 2 (list









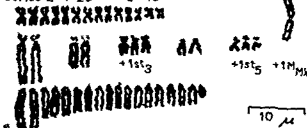


Fig. 7 a–e. Stemline karyotypes from series A–E of primary sarcoma No 4

as less probable The question is of consider able theoretical importance and will be studied further by single cell inoculations

In previous studies (*Mitelman* 1971, 1972) it was demonstrated that the karyotypic changes in primary and metastatic Rous rat sarcomas were non random and suggestive of a sequential *evolution* The initial change was towards a trisomic cell by a gain of 1 t chromosome followed by gains of 1 st_3 and later possibly *1 st* *In the present* *inves* tigation the first and only step in all series of sarcoma No 4 was a gain of 2 t chromo somes No extra t was seen in sarcoma No 2 but the earliest deviating cells of all series of this tumour showed the same submedian marker The origin of the marker could only partly be explained The long arm seemed to be derived from 1 st chromosome whereas the origin of the short arm was uncertain but it might well represent a deleted t chromosome This possibility is mentioned since statis tical analyses of primary and metastatic Rous rat sarcomas (*Mitelman 1971 1972*) *strongly* indicated that chromosome type t was pre ferentially involved in the formation *of mar* kers In all series of passages *of sarcoma No 2 one extra* st_3 *appeared simultaneously with* the marker as the first visible change from normal In sarcoma No 4 the *immediate* second step in the modification of the 44 chromosome karyotype (*+ 2 t*) *was a gain* of 1 st_3 in 3 series and a gain *of 1* st_3 *in 1* series Except for one series which *rapidly* developed to tetraploidy and *one series in* which 1 st_3 initially was lost but later reap peared in a trisomic state the next step in all series of both tumours was a gain of 1 st_3 chromosome Thus supposing that *the sub*median marker of sarcoma No 2 *actually* had originated from a t chromosome *as pro*posed above all 11 series ended with *a gain* of either 1t or 1 st_3 or 1 st *In fact nine of* the series showed gains of all *three* chromo some types From this point on *the* karyo

of sarcoma No 4 diverged from the *pre*scribed *hyperdiploid course* two of these subsequently changed to hypertetraploidy and one to pseudodiploidy It should however, be emphasized that small structural changes, even if systematic, would have passed un noticed in most instances, a change equi valent to the lengthened short arm of 1 st seen in series D of sarcoma No 2, would not have been observed if for example, the long arm had been affected instead

The fact that two series were present in the same animal seemed not to influence the pattern of the chromosomal evolution nor were there any indications that the particular region for the transplant was of any im portance Thus no significant similarities were found within the two series of each animal as opposed to the other *series of the* same tumour

No relationship could be *established* between *any particular chromosome number* or karyotype *and the histopathological* picture of the *tumours However, the general* trend indicated in primary and metastatic *sarcomas* (*Mitelman 1971, 1972*) was con firmed tumours with a normal diploid stem line were highly differentiated whereas hete *roploid tumours regardless of stemline cate gory were anaplastic*

A similar comparative chromosomal inves tigation of the *early in vivo* progression in parallel series derived from the same primary tumour has not to my knowledge previously been published Disregarding long term trans *planted* experimental neoplasms which have

1967) the available information on the early progressional changes in other types of rat tu mours with identical aetiology is scarce In*formation is however*, available about the early progression in 3 other *in vivo* trans planted Rous rat sarcomas (*Levan* 1961) It is of great interest that the results of these original observations agree with the findings of the present study Thus all three early pas*sages 1 1 and 5* respectively showed an *evolution towards stemlines* with 43 chromo

somes by a gain of 1 t chromosome In this context it should also be mentioned that another RSV-SR induced rat sarcoma carried in serial passage since 1961, and also originally studied by *Levan* (1961), apart from numerous other rearrangements still shows (*Mitelman*, unpublished) two of the non-random changes, already observed in the primary tumour and also in the intervening passages (*Kato et al* 1964) one extra t and one extra st_{s} chromosome

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This work was supported by grants from the *Swedish Cancer Society, John and Augusta Persson's Foundation for Medical Scientific Research*, and by grants from the *University of Lund*, Lund, Sweden The excellent technical assistance of Miss *Ing Britt Augrell* and Miss *Birgitta Nordström* and the excellent secretarial help by Mrs *Catrine Dahl* are gratefully acknowledged

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Acta path microbiol scand Section A 80 329-340, 1972

THE FINER INTRAMYOCARDIAL VASCULATURE IN VARIOUS FORMS OF EXPERIMENTAL CARDIAC HYPERTROPHY

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The finer intramyocardial vascular architecture in various forms of cardiac hypertrophy was studied by a combined stereomicro angiographic and histological procedure Significant cardiac hypertrophy was induced by renal hypertension aortic stenosis and swimming exercise The training induced hypertrophy was reversible The myocardial vascular architecture was altered in cardiac hypertrophy There were essential differences in the vascular patterns of training induced hypertrophy on one hand and hypertrophy secondary to hypertension and aortic stenosis, on the other The nature of these differences suggested that training induced hypertrophy was associated with neoformation of vessels in an otherwise structurally unaltered myocardial vasculature, while the other forms of hypertrophy were dominated by a transformation of the preformed vascular pattern In the regression of hypertrophy, which took place during the resting period after training only minor changes appeared in the myocardial vascular pattern

Earlier investigations have been unable to illustrate clearly the structural reactions of the intramyocardial vascular supply in various forms of cardiac hypertrophy *Vannotti* (1936), *Linzbach* (1947, 1960) and *Rakusan & Poupa* (1966) reported an increased capillary supply of the myocardium *in hypertensive* cardiac hypertrophy and in hypertrophy secondary to aortic stenosis, while *Shipley & Wearn* (1937), *Roberts & Wearn* (1941) *Rotta* (1943) and *Hort* (1955) considered that a relative decrease of the capillary supply occurred in such hearts Both a relative increase and decrease of *myocardial* vascular supply in heart enlargement secondary to physical training have been reported (*Thorner* 1936, *Petren et al* 1936, *Tittle et al* 1966, *Leon & Bloor* 1968, *Tomanek* 1970, *Frank* 1950 and *Hakkila* 1955)

By weighing 'corrosion casts' of the coronary artery tree, it has been possible to establish a weight increase, and thus an increased total volume, of the coronary artery system of the hypertrophied heart (*Tepperman & Pearlman* 1961, *Stevenson et al* 1964, *Kerr et al* 1965, 1968) Whether this increased volume depends on a vascular neoformation at the capillary level or on the widening and elongation which the [illegible]

underg

1935 *)*

Vogelberg 1957 and *Baroldi & Scomazzoni* 1965) could not be resolved

The conflicting reports on the structural reactions in the vascular architecture of the hypertrophied heart may depend on the use

Received 23 ix 71

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of different experimental methods, and that none of these methods are entirely ideal It has been demonstrated in a series of earlier works that a combination of stereomicro-angiographic and histological experimental procedures provides good pre requisites for appraisal of the finer vascular architecture of an organ, and of changes therein in association with pathological tissue alterations (e g *Ljungqvist* 1963, *Almgård et al* 1966, *Ljungqvist & Richardson* 1969, *William Olsson et al* 1971) Therefore, we investigated various types of experimentally induced cardiac hypertrophy with this combined stereomicro angiographic and histological technique Rats were used as experimental animals As we were unable to find any complete description of the micro-angiographic picture of the normal rat heart in the litterature, a series of normal rats were examined for comparison

MATERIALS AND METHODS

One hundred and fourteen female Sprague Dawley rats, initial weight 180–200 g were used in the investigation Twenty three rats died during the course of the experiments and were excluded The animals were kept in cages (3–5 in each cage) *They were fed a standard diet containing 0 4 per* cent NaCl and tap water *ad libitum*

Cardiac hypertrophy was induced by three different mechanisms *1* renal hypertension *2* aortic stenosis and *3* swimming exercise At weekly intervals all the rats were weighed and their blood pressures determined by the tail pletysmographic method with the rats under brief ether anaesthesia The initial blood pressure varied between 80 and 130 mm Hg Hypertension was considered to exist at a constant pressure value of 140 mm Hg or higher, provided however, that the initial value had increased by at least 15 per cent

Renal Hypertension

Renal hypertension was achieved in 11 rats by the production of left renal artery stenosis (*Ljungqvist* 1969) in 12 rats by the injection of nephrotoxic serum resulting in glomerulonephritis (*Ljungqvist et al* 1970) and in 12 rats by unilateral radiation nephritis with contralateral nephrectomy (*Ljungqvist et al* 1971)

After the animals were killed the following grouping was made so as to ascertain the dependence of any myocardial vascular changes on the duration of hypertension

1 Thirteen animals with short term hypertension (1–3 weeks)
2 Seven animals with medium term hypertension (*1–3* months)
3 Fifteen animals with long term hypertension (more than 3 months)

Aortic Stenosis

Descending aortic stenosis was induced in 9 animals under ether anaesthesia ad modum *Beznák (1952)* The width of the stenosing silver clip was 0 5 mm The animals were killed after 2 months Normal blood pressures were recorded in all animals by the tail pletysmographic method In five rats the pressure gradient was measured over the stenosis through catheters in the left a femoralis and in the left a carotis The pressures were 155 ± 15 mm Hg in the carotis and 130 ± 10 mm Hg in the femoralis which is in good agreement with the pressure gradient of 25 mm Hg recorded by *Morkin & Ashford* (1968) on similarly treated rats

Swimming Exercise

Sixteen animals were trained in a 150×80×70 cm large, waterfilled plexiglas bath at a water temperature of about +28° C The animals swam 1 hour per day 6 days per week for 3 months During each training period it was checked that the animals actually swam and did not merely float They were markedly inactive for 2 3 hours after every training period No hypertensive values were *registered* The *animals* were divided into 2 groups

1 Eight animals which were killed after 3 months training
2 *Eight animals which were killed after* 3 months training followed by 2 months rest

Control Animals

The following controls were used

1 Eight entirely untreated rats
2 Thirteen sham treated rats In three of them the *operative procedures* for left renal artery stenosis were performed but no silver clips applied The remaining 10 rats were given intravenous injections of NaCl and were intended *primarely as controls for the* glomerulonephritic animals
3 *Ten rats in which unilateral* renal artery stenosis was induced without leading to hypertension

On termination of the experimental *period* the rats were anaesthetized with ether whereupon the sternum was split and the heart exposed *Five ml* of a 10 per cent aqueous suspension of fine grain barium sulphate (Micropaque) were injected into the left atrium The animals *died during* the injection Thereafter a catheter was inserted in re-

trograde direction into the ascending aorta, and the vessels to the head and front extremities were ligated The Micropaque suspension was injected via the catheter for about 60 minutes The infusion pressure was checked continuously by a manometer, and increased gradually from 30 to 110 mm Hg During the initial phase of the injection heart activity with evident ventricular contractions was restored for about 5 minutes in almost all cases

On termination of injection, the heart was removed and weighed In order to ascertain whether cardiac hypertrophy had occurred in the experimental animals the heart weight/body weight ratio was determined for each animal, for practical reasons multiplied by factor 1000

After weighing the heart was fixed in 10 per cent formalin for 24 28 hours for combined stereo micro-angiographic and histological examinations according to the previously described method (*Ljungqvist* 1963) This involved, in brief, that *1 5 mm thick transversal slices of the ventricular* segment were cut in a series from the atrio ventricular level to the apex The slices were then embedded in a mixture of bee s wax and paraffin after which they were cut in 100 800 μ thick blocks The latter were exposed by stereoscopic technique in a fine focus x ray tube and on a fine grained photographic emulsion (Kodak MRP) The micro angiograms were studied in a specially constructed stereomicroscope

The micro angiographed blocks were re embedded and cut in thin sections which were stained either with haematoxylin eosin or with van Gieson s connective tissue stain counterstained with Weigert's elastin staining method These sections were examined in an ordinary light microscope

RESULTS

Table 1 shows that a significant heart-weight increase, as compared with the entirely normal rats (Control Group 1), occurred in the hypertensive rats, in the rats with aortic stenosis and in the swimming trained rats which did not have any resting period after training The source of error in these values, however, may be relatively great, as contrast medium was injected into all the hearts Thus, it is of interest that basically the same effect on the heart-weight was obtained in experiments which were performed in parallel, and contrast injections were not given (Table 2) The material on which this table is based is included in a later study

Normal Vascular Pattern

The left ventricle wall contains large subepicardially located arteries, which send major vessels into the myocardium These vessels are of two types One type, "A ves-

TABLE 1 *Heart Weight × 1000/Body Weights Ratio (R) and the Terminal Blood Pressure (B p) in Rats*

Group	Dur	No of rats	R	B p
Control 1	3 mos	8	5 1±0 6	115+10
Control 2	3 mos	13	5 0±0 8	115±15
Control 3	3 mos	10	5 7±0 2	120±10
Hypertens	1–3 wks	13	6 3±1 3**	180±15
Hypertens	1–3 mos	7	6 6±0 8***	170±15
Hypertens	3 mos	15	6 5±1 5**	170±30
Ao sten	2 mos	9	6 0±0 3**	105±10
Swim 1	3 mos	8	7 0±1 0***	100±15
Swim 2	3+2 mos	8	5 6±0 7	110+15

The Table shows the heart weight × 1000/body weight ratio (R) and the terminal blood pressure (B p) in rats with renal hypertension of various durations (Dur) in rats with aortic stenosis (Ao sten) and in rats subjected to swimming exercise only (Swim 1) and swimming exercise followed by a resting period (Swim 2) Untreated rats (*Control 1*), sham operated rats (Control 2) and rats subjected to renal artery stenosis operation not followed by hypertension (Control 3) constitute control groups All hearts were injected with contrast medium before [illegible]

TABLE 2 *Heart Weight × 1000/Body Weight Ratio (R) and the Terminal Blood Pressure (B p) in Rats*

Group	Dur	No of rats	R	B p
Control 1	3 mos	7	3.5±0.3	95±10
Control 2	5 mos	8	3.6±0.3	85±15
Control 3	3 mos	17	3.8±0.4	130±20
Hypertens	1 wk	10	4.8±0.9**	170±15
Hypertens	3 mos	8	5.0±0.7**	180±25
Ao sten	2 mos	8	4.2±0.5**	120±10
Swim 1	3 mos	8	4.3±0.3**	120±20
Swim 2	3+2 mos	9	3.8+0.3	120±15

The Table shows the heart weight × 1000/body weight ratio (R) and the terminal blood pressure (B p) in rats with renal hypertension of various durations (Dur), in rats with aortic stenosis (Ao sten) and in rats subjected to swimming exercise only (Swim 1) and swimming exercise followed by a resting period (Swim 2) Untreated rats (Control 1), sham operated rats (Control 2) and rats subjected to renal artery stenosis operation not followed by hypertension (Control 3) constitute control groups The hearts were not injected with contrast medium The figures are means ± SD When compared with the untreated and sham treated controls a significant increase in heart weight was recorded in the hypertensive rats, in the rats with aortic stenosis and in the rats killed immediately after swimming exercise (** $0.001 < p < 0.01$)

sels", abruptly split up at sharp angles into 3–7 considerably thinner branches (Fig 1) These pass over in arterioles and capillaries which usually undergo dicotomic, U-shaped divisions and thus create capillary networks of parallel running interanastomosing vessels (Fig 2) The other type, "B vessels' depart from the coronary arteries at angles of 50–90° and continue without division or marked loss of diameter through the left ventricle wall, at an angle of about 30° in relation to the plane of sectioning (Fig 1) They end in the subendocardial capillary plexus and the , networks of the papillary musculature by an abrupt sub division into a number of small branches (Fig 3), some after having pursued a terminal loop like course (c f *Estes et al* 1966 *Farrer-Brown & Wartman* 1967)

The subendocardial plexus occupies about 10–15 per cent of the entire thickness of the *left ventricle wall*, whereas the subepicardial plexus is much thinner

No interarterial or interarteriolar anastomoses were found in the myocardium A small number of spiralling arterioles were found in the outer third of the left ventricle wall and in the angle between the wall and septum, this feature was particularly frequent in the hearts from Control Group 3

The right ventricle wall is in appearance similar to the left, but contains no vessels of the 'B type' Furthermore the subendocar

Fig 1 Micro angiogram of left ventricle wall in normal rat heart showing A type vessels (*smaller arrows*), *which give rise to thinner intramyocardial* vessels and a B-type vessel (*larger arrow*), which runs undivided and with unchanged calibre through the ventricle wall towards the subendocardial plexus (not visible on picture) × 35

Fig 2 Micro-angiogram of left ventricle wall in normal rat heart showing the capillary networks of uniform meshes which are *orientated in a fashion* of parallel planes U shaped branchings are seen (*arrow*) × 90

Fig 3 Micro-angiogram of left ventricle wall in normal rat heart showing the terminal portion of a B-type vessel (*arrow*) On reaching the subendocardial plexus the vessel divides into *smaller* branches which join the capillary *networks of the* subendocardial plexus and papillary musculature × 35

Fig 4 Micro-angiogram of left ventricle wall in normal rat heart showing venous drainage of so-called turnip-root character which empties into epicardial veins × 35


1
2
3
4

dial plexus is very thin and the capillary network in the entire wall is sparser

The central part of *the* septum is supplied by a major vessel, which splits up into several branches in the upper part of the septum These branches descend far to the right in the septum towards the apex region (c f *Halpern* 1957, *Farrer Brown* & *Rowles* 1969) The ventral and dorsal areas of the septum are supplied by branches which originate from arteries in neighbouring parts of the ventricle walls, mainly the right Towards the left ventricle the septum has a subendocardial plexus of the same appearance as in the actual left ventricular wall To the right, the subendocardial plexus of the septum is very thin, even thinner than in the right ventricle wall The inner part of the septum displays a capillary pattern identical with that seen in the left ventricle wall

The venous drainage forms so called turnip roots (*Brown* 1965) in the entire left ventricle, septum and right ventricle and empties into epicardially located veins (Fig 4)

Normal Histological Picture

The main subepicardial arteries are relatively thin walled with an obvious elastic component and a thin muscular coat The larger intramural vessels are relatively thick walled with a prominent muscular component The muscle fibres of the myocardium course in different layers with characteristic orientation of the fibres in each layer In transverse sections of the heart the fibres are crosssectioned subepicardially in the left ventricle and to the right in the septum In the middle third of the left ventricle and septum the fibres are usually longitudinally sectioned while the inner third with the subendocardial plexus and the papillary muscles again have cross sectioned fibres The same conditions apply in the right ventricle but there the layers are considerably thinner

Changes During Cardiac Hypertrophy

In the hypertensive rats micro-angiographic and histological alterations in the hearts were related to the *duration of the* hypertension but not to its mode of induction The possible role of the height of the blood pressure level in the hypertensive animals is difficult to evaluate due to fluctuations in the blood pressure during the experimental period and the variations in the duration of the hypertension

Long term hypertension The septum arteries and their branches are dislocated to the right There is an abundance of spiralling arteries and arterioles in the left ventricle wall and septum and particularly in the angle between the septum and left ventricle (Fig 5) Spiralling is also seen at the capillary level The U shaped branchings are widened and the capillary meshes oval or rounded with a considerable irregularity and poor parallelity in their patterns (Fig 6) There is no definite change in density of the capillary network In segments of the left ventricle wall, extending from the epicardial to the endocardial surface, contrast filled vessels are few or absent, this feature is referred to as segmental filling defects (Fig 7) The vascular pattern of the right ventricle wall is normal apart from the presence of some spiralling arterioles

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Fig 5 Micro-angiogram of left ventricle wall of hypertrophied rat heart (hypertension > 3 months) showing intensely spiralling arterioles (*arrows*) × 35

Fig 6 Micro angiogram of left ventricle wall of hypertrophied rat heart (hypertension >3 months) showing more irregular capillary patterns than normal with widened U shaped branchings (*arrows*) and abolished parallelity of the capillary meshes (c f Fig 2) × 90

Fig 7 Micro angiogram of left ventricle wall of hypertrophied rat heart (aortic stenosis) showing characteristic segmental filling defects which extend from the epi to the endocardial surface × 15

Fig 8 Micro-angiogram of left ventricle wall of hypertrophied rat heart (swimming exercise) showing a dense and abundant anastomosing capillary network with well retained parallelity (c f Figs 2 and 6) × 90


5


6


7


8

The walls of both spiralling and non spiralling arteries and arterioles display marked thickening of their muscle layers with increased numbers of nuclei The left ventricle wall is clearly thicker than normal the cross sectioned middle third layer being particularly thickened Moderate fibrosis is present in the left ventricle wall and septum

Short term hypertension There are fewer spiralling vessels than in rats with long term hypertension The U shaped branchings are normal A tendency towards abolition of the parallelity of the capillary meshes is observed There is no definite change in the density of the capillary networks

The coronary arteries are histologically normal There is some thickening of the muscle layer and increase in number of nuclei in the walls of the large branches of the main coronary trunks The spiralling arterioles are of normal histological appearance The left ventricle wall is moderately thickened due to an increased width of the middle third layer The findings in the right ventricle are similar to those in the controls

Medium term hypertension Findings in these rats represent transitions between changes observed in the left ventricle wall during short and long term hypertension The pattern in the right ventricle wall is similar to that in the controls

Aortic stenosis The septum arteries (or their branches) are dislocated towards the right There is an abundance of spiralling arteries in the left ventricle wall and septum Arteries and arterioles are thicker and more well filled with contrast medium than in all other groups Arterioles and capillaries divide in a more widened U shaped fashion Like in long term hypertension there is a marked irregularity and a poor parallelity in the capillary pattern with numerous short intercapillary anastomoses at all levels but no definite change in density of the capillary network There is a large number of spiralling pre capillary and capillary vessels Segmental filling defects are frequent throughout the left ventricle wall (Fig 7) Occasionally spiralling arterioles are encountered in the right ventricle wall but otherwise the conditions in this wall are normal

The coronary arteries and larger intramural arteries and arterioles are slightly dilated and their walls are of normal thickness or possibly thinner than normal Thus no increased thickness of the muscle wall is present Spiralling vessels show a normal wall structure The left ventricle wall and the septum are thinned as a result of a reduction of the middle third layer A slight degree of diffuse fibrosis is present in the left ventricle wall and septum

Swimming Exercise for 3 Months

The septum arteries and their branches are dislocated towards the lumen of the right ventricle No spiralling vessels are observed The intramural arteries do not course at the 30° angle in relation to the plane of sectioning but parallel to this plane The U shaped branchings are normal in appearance or flattened and the capillary meshes elongated The capillaries are of normal width The capillary networks appear denser than normal with a preserved parallelity of the meshes (Fig 8) This capillary network extends throughout the major portion of the left ventricle wall and septum and is accompanied by a relative or possibly absolute decrease in thickness of the subendocardial plexus There are no segmental filling defects The conditions in the right ventricle are similar to those in the left

Coronary arteries larger intramural vessel and arterioles are of entirely normal histological appearance The left ventricle wall like the septum is considerably thickened due to a widening of the middle and inner third layers No interstitial fibrosis is noted

Swimming Exercise for 3 Months + Rest 2 Months

The alterations are similar to those observed after swimming exercise for 3 months with three exceptions (i) the relative or absolute decrease of the subendocardial plexus is not quite as pronounced (ii) spiralling

vessels occur, and these are somewhat more frequent than in the controls, and (iii) the increased density of the capillary vasculature appears less pronounced

The histological picture is similar to that after swimming exercise for 3 months, except for a reduction of the thickened middle and inner third layers However, some thickening of the left ventricle wall appears to persist

DISCUSSION

It appears from a review by *Badeer* (1964) that many different methods are used to induce cardiac hypertrophy in experimental animals Naturally, it would be of interest to *study the intramyocardial vascular architecture* in these various forms of hypertrophy, particularly as in this work we found different patterns of reaction in the vascular tree in the various kinds of hypertrophy we studied However, the purpose of our investigations was restricted to a comparison between the reactions in pathological cardiac hypertrophy secondary to arterial hypertension, 'pathological' cardiac hypertrophy secondary to aortic obstruction and "physiological' hypertrophy induced by swimming exercise

An increase in heart weight is the usual criterion for cardiac hypertrophy and is established most reliably as an increase in the heart weight/body weight ratio, provided, of course, that the pathological condition in the experimental animals has not led to any dramatic, terminal change in body weight No such change in body weight was recorded in our experimental groups. On the other hand, a retarded weight increase during the rest period after concluded swimming exercise was established and this can be accepted as an indication that the training was effective (*Mayer et al* 1954, *Bloor & Leon* 1970, *Tomanek* 1970) This experimental group was also the only one which did not display any definite cardiac hypertrophy As the animals, which were killed immediately after termination of training showed pronounced cardiac hypertrophy, with greater heart weight than any other group, experimental evidence was obtained that cardiac hypertrophy induced by physical training can be reversible

The present investigation has shown that definite changes occur in the myocardial vascular architecture in experimentally induced cardiac hypertrophy The essential observation was, however, that different changes in physiological and pathological cardiac hypertrophy existed In contrast to hypertension and aortic stenosis induced cardiac hypertrophy, swimming exercise-induced hypertrophy demonstrated i) no segmental filling defects, ii) no widening of the U shape of the arteriolar and capillary branchings, iii) no spiralization of the vessels and iv) a reduced *thickness of the subendocardial plexus network* Furthermore, the capillary network appeared micro angiographically denser in the swimming exercised rats than in the rats with hypertension and aortic stenosis

In earlier investigations of the intramyocardial vascular reaction in cardiac hypertrophy, attention has mainly been concentrated on evaluation of the capillary density, as this may be considered the morphological basis for myocardial nutrition Conflicting results have been obtained Thus, in swimming training or other forms of training induced hypertrophy, *Petrén et al* (1936), *Tittle et al* (1966), *Leon & Bloor* (1968) and *Tomanek* (1970) reported an increased capillary supply of the myocardium, while no such change was established by *Frank* (1950) *Hort* (1951) and *Hakkila* (1955) In investigations of this nature, a determination of the capillary/muscle fibre ratio in histological sections was generally used This method is obviously more reliable than a mere estimation of the density of the capillary networks on micro-angiograms However, in our investigation the differences in the density of the capillary networks in physiological and pathological hypertrophy were pronounced to a degree suggesting that the prerequisites for adequate nutrition must have been considerably better in physiological hypertrophy, in the pathologically hypertrophied hearts,

the capillary supply even appeared so poor that segmental filling defects occurred

Incomplete filling of injection medium may represent artefacts (*Ljungqvist* 1963) In all probability, however, this was not the case with the above mentioned segmental filling defects of the myocardium, as they were a pervading feature in the aortic stenosis operated animals and in those with hypertensive periods exceeding 3 months, but were never observed in the other groups Since it can be assumed that the oxygen supply within the incompletely vascularized areas was deteriorated, the change may be the morphological basis for the diffuse myocardial fibrosis observed in the rats with aortic stenosis and long-term hypertension

The differences in the capillary branching patterns in physiologically and pathologically hypertrophied hearts may be explained by the above-discussed differences in the reaction of the capillary vascularization The reason why the vascular branching assumes a widened U-shape would appear to be that the enlarging muscle fibres displace the vessels If so, this would naturally occur in all forms of hypertrophy That the change was not observed in the physiologically hypertrophied hearts may, however, be explained by a formation of new capillaries which themselves assumed a branching pattern adapted to the increased fibre thickness and which led to a micro-angiopraphically denser capillary vasculature in which the preformed and altered vessels were partly obscured

Spiralling of originally straight vessels has been observed earlier in pathologically altered organs (*Ljungqvist* 1963) It was pointed out that, in theory, two principal mechanisms may lead to this change, on the one hand the vessel may assume a spiralling course when it grows in length between fixed end points as in a so called collateral adaptation and, on the other, the change may be the result of a shortening of the distance between the end points of the vessel such as occurs in fibrous contraction or atrophy of the surrounding parenchyma Vessel spiralling in the pathologically hypertrophied hearts may be explained by a growth in length of the myocardial vessels parallel with the increase of the muscle mass The slight but evident spiralling which was observed in several of the normotensive animals with renal artery stenosis, may be explained on the same grounds, it is probable that these animals had experienced a transient hypertension which is not uncommon in unilateral renal artery stenosis This possibility is also supported by the somewhat higher heart-weight in this group in comparison with the other control groups, which is particularly evident in the injected material (Table 1)

The micro-angiographically dense vascularization of physiologically hypertrophied hearts suggested that the increased muscle mass in these was supplied by newly formed vessels This is consistent with the absence of spiralling vessels in these hearts, indicating that growth of pre existing vessels did not make a major contribution to the increased vascular supply In accordance with the explanations given above spiralling appeared, however, when the hypertrophied heart muscle reassumed a normal size during the period of rest following the swimming exercise

The reduction in thickness of the subendocardial plexus which was observed in training induced hypertrophy, is probably an expression of reduction in the thickness of the subendocardial tissue zone secondary to enlargement of the neighbouring myocardial muscle layers That this change was not observed in the other forms of hypertrophy may be due quite simply to the fact that the degree of hypertrophy in these forms was less than in the swimming exercised animals

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This study was supported by the *Swedish Medical Research Council* (Project No B 69 – 12 x – 716 04 A) and the *Swedish National Association against Heart and Chest Diseases*

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Acta path microbiol Scand Section A 80 341–344, 1972

THE INTENSITY OF ULTRASOUND IN THE UTERUS DURING EXAMINATION FOR DIAGNOSTIC PURPOSES

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The report here submitted renders an account of the results obtained by measurements of the damping of ultrasound during passage from the abdominal surface to the uterine cavity in women in the reproductive age Ultrasound was emitted from a transmission transducer on the abdominal surface and received by a specially designed miniature transducer introduced into the uterine cavity The damping was found to average 2 5 dB

A comparison of the biological effects of ultrasound in experimental animals and man requires knowledge about the damping in all tissues between the ultrasound transducer and the tissue to be examined

Knowledge of the factors effect emitted from the transducer, coupling coefficient, and damping facilitates calculation of the intensity with which ultrasound acts on the tissue

The object of the present work is to measure the damping during passage through the tissues interposed between the abdominal surface and the uterine cavity in women in the reproductive age the equivalent damping in the mouse (*Bang and Northeved* 1970) has previously been analysed in an experimental teratological series

METHODOLOGY

With the aim of measuring the damping during passage from the abdominal surface to the uterine cavity a transmission transducer (2 25 MHz) was placed on the abdominal surface while a receiver transducer was introduced into the uterus

A 12 mm barium titanate crystal (PZ 11) mounted on turbax was used as transmission transducer

The previously described miniature transducer (*Bang and Northeved* 1970) served as receiver transducer The crystal in the latter is 1 mm thick its diameter measuring 1 5 mm

In order to escape errors in measurements owing to a poor centering of the two transducers it has been necessary to have a fixture designed in which the two transducers are kept in such position that the centres of the two crystals no matter the distance between transducers converge on one axis The fixture is depicted in Fig 1 It is composed of a 12 mm stainless steel tube (1) to which the two holders for transducers are fitted The holder (2) for the miniature transducer is composed of a 140 mm long stainless steel tube to which another tube is welded the latter being of the same shape as a Hegar dilator no 6 and thus permitting introduction into the uterus The miniature transducer pointing towards the transmission transducer is cast integral into the free end of this tube

The holder (3) for the transmission transducer is composed of a 12 mm tube to which a plain bearing is welded at a gradient adjusted to the slightly curved Hegar dilator no 6, thereby per

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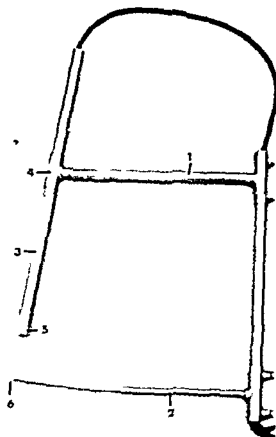


Fig 1 This fig shows the fixture with the transmission transducer (5) and the receiving transducer (6) For further information please see the text

mitting that the transmission transducer fitted to one end of a 14 mm stainless steel tube can be displaced in the axial plane in relation to the miniature transducer

The ultrasonic equipment is of Krautkramer USIP 10 type which on account of its precise attenuator has been found particularly well suited for measurements of damping

Owing to the pattern of radiation emitted from the ultrasonic transducers, comprising a short range as well as a long range (e g *Sunden* 1964), damping has to be determined by relative measurements In the routine, a characteristic of the damping is recorded as the function of the distance between the two transducers while the latter are submerged in decontaminated distilled water at 20° C In other media damping may be determined by a comparison of that to be read on the ultrasonic apparatus and the characteristic measured in water

The equipment was controlled in water prior to and after measurement of damping in women

The measurements were carried out on a series of women who in order to have antigon changed met in the out patient ward in the gynaecological obstetrical department G of Gentofte Hospital Groundnut oil served as coupling medium between the transmission transducer and the abdominal surface The receiver transducer in the uterus was coupled directly to the humid uterine mucosa (women in whom measurements were performed were not allowed to void urine later than two hours prior to examination)

RESULTS

The results obtained appear from Table 1

The ratio of relative damping in dB to

TABLE 1 *Damping Measurements*

No of patients	Relative damping in dB	Relative distance between transducers in cm	Relative damping in dB cm – damping in dB/cm
1	0	3	0/3 = 0
2	2	4	2/4 0.5
3	4	4½	4/4½ 0.9
4	4	4	4/4 = 1
5	2	4	2/4 0.5
6	2	3	2/3 0.67
7	4	4	4/4 = 1
8	2	2	2/2 1

The table records the damping during passage from the transmission transducer on the abdomen to the receiver transducer in the uterus also the distance between the two transducers in cm is stated The relative damping per cm of tissue is recorded in the third column Damping in water *being insignificant* about 1000 times less than that in tissue, the actual damping to be encountered in practice will equal the relative damping

measurements in water is recorded in the first column

The second column records the distance between transmission transducer and receiver transducer during measurements in the women The distance is read on a scale engraved into the tube to which the transmission transducer is fitted

The third column reflects the relative damping in dB per cm of tissue The actual damping encountered in practice will equal the relative damping damping in water being insignificant as compared with that in tissue, namely about 1000 times as low (*Hill* 1968)

DISCUSSION

The intensity of the ultrasound to be produced in the uterus can theoretically be calculated on the basis of the effect known to be emitted from the ultrasonic transducer and cognition of the factors damping and reflexion in tissues through which the ultrasound has to pass (*Hill* 1968) These calculations imply a knowledge of the distance between the transmission transducer and the uterine cavity the tissues and organs through which the ultrasound is to pass must also be well defined Damping of ultrasound is highly dependent on two facts namely whether the uterus is in direct contact with the abdominal wall or whether intestines and/or the urinary bladder are interposed since damping in spaces filled with air is about 10^5 times as high as that in bladders filled with fluid Consequently examinations of women in early stages of pregnancy (6th to 10th week) have to be performed while their urinary bladders are filled owing to the intense damping in intestines filled with air

Exact data on these features are not readily accessible It has been attempted by means of X rays to have the distance between the uterus and the abdominal surface determined (*Harwich* 1968) Such distance may be judged by ultrasonic scanning but the thickness of the various tissues cannot be reliably ascertained

With a view obtaining an exact standard of the damping it has been preferred to perform the above discussed direct measurements in women in the reproductive age

The methodology used here has been elaborated on the basis of findings in preliminary experiments in which the measurements were carried out according to two different principles

The transmission transducer and the receiver transducer first used did not comprise mechanical fixation These primary transducers were randomly adjusted in the axial plane and subsequently manipulated until deflexion on an oscilloscope was maximal This method was rejected because the results provided were not reproducible

Later on another method was introduced according to which the transmission and receiver transducers were arranged in highly stable fixtures in which the transducers were kept in the axial plane even though their mutual distance might be changed The results thus obtained were reproducible for which reason this principle has been used Transducers as well as fixtures were controlled in water bath Measurements were performed over distances of 2 4 8 and 12 cm Thus it was established that the central part of the ultrasonic field was rotatory symmetrical and also that the transducers were adequately fixed and that the lines through the centres of the two transducers converged because it emerged from control measurements that the maximal deviation ranged at ± 0 53 dB

Owing to the hazards of infection involved whenever instruments are introduced into patients and also to the fact that measurements showed only minor scatter, it seemed reasonable to abstain from measurements other than the eight mentioned above

The damping of 0 dB observed in patient no 1 and recorded in the table may be interpreted as a damping of an order of magnitude commensurate with the precision of the equipment i e ± 1 dB

According to the results thus obtained damping of ultrasound during passage from the abdominal surface to the uterine cavity

has been found to be rather insignificant and hence it may be ignored in valuations of the intra uterine energy level during examinations by ultrasound for diagnostic purposes

The average degree of damping (2 5 dB) was found to be of an order of magnitude identical to that previously observed in the mouse (1 6 dB) (*Bang and Northeved* 1970) Whenever the intra uterine energy levels in the mouse and man are to be compared it will not be unreasonable to ignore the difference in damping in the various tissues

In the fields of gynaecology and obstetrics reasons of security require that such damping is ignored during clinical examinations by which to evaluate the energy level

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Acta path microbiol scand Section A 80 345 350, 1972

LIGHT AND ELECTRON MICROSCOPICAL STUDIES OF RECTAL BIOPSIES IN CYSTIC FIBROSIS

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Light microscopy of rectal mucosa from patients with c. f. could not confirm the occurrence of morphological alterations which have prior been described as characteristic of c. f. Neither could electron microscopy reveal alterations in the fine structure of goblet cells, principal cells and endocrine cells

Cystic fibrosis of the pancreas (c f) is a hereditary diffuse abnormality of secretory epithelial cells in mucous as well as serous glands This is manifested by abnormally viscous mucous secretions and disturbed levels of electrolytes in serous secretions *Bodian* (1953) has given the most detailed description of light microscopy findings in secretory epithelial cells in post-mortem tissues from children with c f

Light microscopy examination of rectal mucosal biopsies has been used as an aid in the diagnosis of c f (*Parkins et al* 1963, *Eidelman et al* 1964 *Jabro et al* 1966) Common findings in these studies were goblet cell hyperplasia distended, prominent goblet cells and widely distended crypts, packed with mucus The mucus at time appeared lamellated

The goblet cells are simple unicellular glands secreting a sulfated carbohydrate protein complex By means of radioautography their secretory mechanism has been clarified (*Neutra et al* 1969) The protein part is primarily synthesized on ribosomes at the base of the cell It then moves to the Golgi complex where carbohydrate is added to the protein In the Golgi saccules sulfate is further added to the carbohydrate part of the new glucoprotein As the saccules are filled with glucoprotein they bud off the saccules stack as granules which then migrate to the luminal cell membrane and release their contents The present work was primarily undertaken in order to study whether the production of abnormal secretions was reflected in the fine structure of the rectal mucosa

A second purpose was to test the utility of electron microscopy and light microscopy on thin sections of rectal biopsies as a diagnostic method in c f

MATERIAL AND METHODS

Biopsies of rectal mucosa were taken from 20 children. The biopsies were coded and estimated blindly Control of hæmoglobin concentration, clotting time and bleeding time the number of patelets and prothrombin concentration was routinely performed By rectoscopy the mucosa was found nor

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mal in all cases. The biopsies were taken with biopsy forceps 6–7 cm from the anal region. The specimens were flattened cut surface down onto a cardboard and fixed in iced 3 per cent glutaraldehyde buffered with Na-cacodylate to pH 7.4 immediately after excision. The specimens were cut into wedge-shaped blocks according to *Pittman et al.* (1966), post-fixed in OsO_4 1 per cent, dehydrated and embedded on edge in epon or araldite. In this way well orientated 1 mu thick sections from 99 blocks were studied by light microscopy, 5 biopsies being excluded because improper orientation, trauma and degeneration of the mucosa. The material then comprises biopsies from 15 children in all, 81 blocks. One mu thick sections were stained with toluidine blue and periodic acid Schiff (PAS) and used to locate the area to be trimmed for ultrathin sectioning in various parts of the crypts. One mu thick sections for light microscopic examination were obtained before as well as after thin sectioning. Sections were cut on a Reichert OmU2 ultramicrotome and thin sections were examined with a JEM 17 electronmicroscope after staining with uranyl acetate and lead citrate.

After blind review of all biopsies the results were compared to the code. Seven children had c.f. and eight children in which the diagnosis of c.f. was excluded served as controls. The children with c.f. had chronic obstructive pulmonary disease, were given pancreatic enzyme replacement therapy because azotorrhoe and steatorrhoe and all had abnormal sweat test.

RESULTS

By the *light microscopic examination* of 1 mu thick sections no characteristic differences between biopsies from patients with c.f. and the "normal" controls could be demonstrated. Roughly estimated the number of goblet cells in various parts of the crypts was equal and the goblet cells were not swollen or elongated in biopsies from patients with c.f. In biopsies from both groups the only structural changes of the crypts were moderate dilatation often combined with slight flattening of the epithelial cells but never with large content of mucus. The mucus never appeared more dense in biopsies from patients with c.f. than in biopsies from the "normal" controls (Fig. 1).

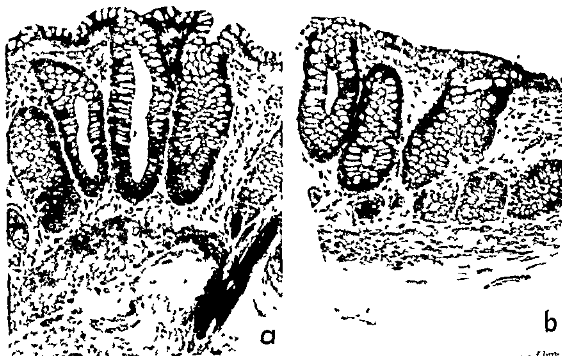


Fig. 1. Light micrographs of sections of epon embedded biopsies of rectal mucosa (a) in cystic fibrosis and (b) normal. Toluidine blue × 100.

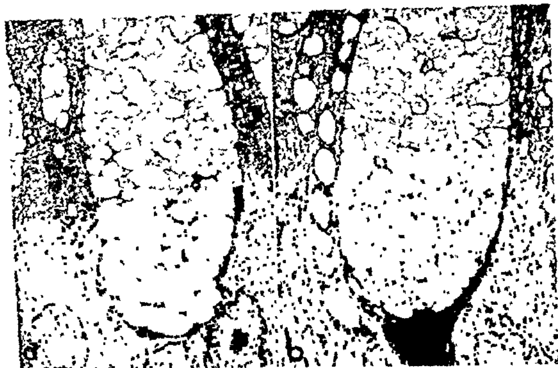


Fig ... normal (× 3,200)

I ... study of the rect ... attention was only given to the lining epithelium Three types of cells previously described (*Pittman et al* 1966, *Lorenzsonn et al* 1968) were found The principal cells the goblet cells and the endocrine cells

The luminal surface of the principal cells was characterized by microvilli coated with a finely fibrillar mucosubstance (glucocalyx) The apical cytoplasm contained vesicles, the limiting membrane of which resembled the surface membrane In biopsies from both groups invaginations of surface membrane indicating pinocytosis could be seen Microvilli and glycocalyx appeared normal in all biopsies

We did not in any of the biopsies find changes in the goblet cell organelles known to be responsible for the production of mucus (Fig 2 3, and 4) The mucus in saccules as well as in the granules was finely fibrillar The mucus secreted from the goblet cells was more coarse than the intracellular mucus and contained varying amount of cell organelles No differences between the extracellular mucus in biopsies from patients with c f and the "normal" controls could be observed

Endocrine cells of the human gastrointestinal tract have been described (*Capella et al* 1969, *Forssmann et al* 1969, *Pearse et al* 1970) Based upon the fine structure of the secretory granules these authors differentiated between seven types of endocrine cells In the present study two types of endocrine cells were observed, morphologically identical with two types of cells found in the human jejunum and called *enterochromaffin cells* and large granule cells respectively The cells were counted in various parts of the crypt They were particularly located in the basal and intermediate parts Biopsies from patients with c f and from "normal" controls showed endocrine cells of identical ultrastructure and the cells were found in the same parts of the crypts in equal number in the two groups of biopsies

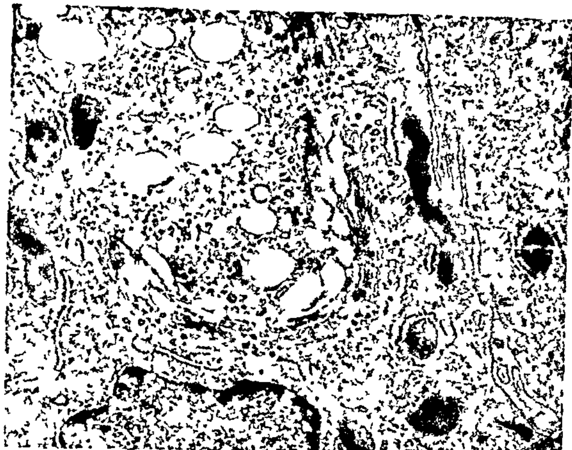


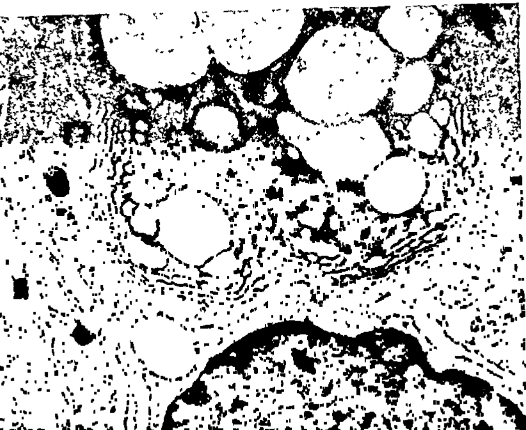
Fig 3 Electron micrograph of a goblet cell Golgi komplex from the epithelium of rectum of a child with cystic fibrosis (× 22 800)

DISCUSSION

The mucus secreted from the goblet cells in patients with c f was suggested to be more viscous than normal mucus However histochemical studies have failed to demonstrate any qualitative changes specific to this disease (*Hagnet et al* 1960 *Lev et al* 1965 *Johansen et al* 1969) although an increase in the concentration of protein and weak acid-groups and of sulphated and sialic acid containing mucosubstances has been found *Johansen et al* (1968) have put forward the hypothesis that the newly synthesized material is insufficiently diluted during elaboration of the final secretion within the cell, owing to inhibition of fluid movement from the extracellular space

The most extreme involvement of the gastrointestinal glands is found in cases of c f with meconium ileus m i) (*Thomadis et al* 1963) Intestinal obstruction in patients with c f of varying ages has been reported (*Beck et al* 1966), and has been described as meconium ileus equivalent (m i æ) (*Jensen* 1962)

Another frequent symptom in c f patients is rectal prolapse The causes of the rectal prolapse tendency has not been explained but it is tempting to see a connection between the changes mucus and the tendency of not only m i and m i æ but also of rectal prolapse. One of our patients developed intestinal obstruction a few days after rectal biopsy has been taken This patient had previously had rectal prolapse Our light microscopic observations on rectal biopsies from patients with c f differed from those previously reported (*Parkins et al* 1963 *Eidelman et al* 1964 *Jabro et al* 1966) We did not find any of



ıg 4 Electron micrograph of a goblet cell Golgı komplex from the epıthelium of rectum of a "normal' hıld (× 18,720)

he features considered as characterıstic of f This could be due to dıfferences between our materıal and those previously reported, ın he degree of gastrointestinal involvement and medicinal treatment at the tıme of the rectal biopsies Thus, none of our patients have had m ı but neither ın the rectal biopsy from the patıent with m ı æ dıd we find any changes

Our findings that changes previously considered characteristic of c f are not specific are ın accordance with the observations of sımilar changes ın "normal" controls (*Johansen et al* 1969) and ın biopsies from patients with rectal disease proper (*Gear et al* 1968)

Electron microscopic examination disclosed no alterations ın the fine structure of the epithelial cells of the rectal mucosa Electron microscopic examination of small intestine biopsies from 21 patients with c f (*Frey et al* 1964) showed a coarse fibrillar substance covering large areas of the biopsy specimens from the patients with c f who had steatorrhoe It was not observed ın normal biopsies or ın one patient without steatorrhoe We dıd not find the above mentioned changes neither dıd our periodic acid-Schiff staın of 1 mu thick plastic sections indicate any thickened mucinous cover of the striate border All our patients were ın therapy with enzymes (pancreatin, Rosco) at the time when biopsies were taken, and none of them had steatorrhoe

In the present light- and electron microscopic study, thus, no alteration was found ın the rectal mucosal structure This may be ascribed to the absence ın our materıal of patients with m ı or steatorrhoe and only one patient with m ı æ.

This work was supported by a grant from *The Danish Cystic Fibrosis Research Foundation*

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Acta path microbiol scand Section A 80 351 356, 1972

RENAL CORTICAL NECROSIS

An Evaluation of the Possible Relation to the Shwartzman Reaction

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In the course of thirteen years, 5994 postmortem examinations were done among which six cases of renal cortical necrosis were found These are classified according to micro scopical changes Only three were registered as complications to obstetric diseases The manifestation of microfibrin thrombosis is found to bee an essentiel factor in the patho genesis of the condition According to the similar findings in the generalized Shwartzman reaction the pathogenesis of renal cortical necrosis is discussed and it is presumed that this condition is confined to a common mechanism, through a varying clinical picture and apparently different pathogenesis

The term bilateral cortical necrosis is reserved for an uncommon most often fatal, condition which *may* be clinically well defined, but the establistment of a definite diagnosis requires biopsy or postmortem examination Macroscopically the condition is characterized by bilateral diffuse necrosis of the renal cortex The cut surface of the kidneys shows varying necrosis of the cortex which appears pale yellow, contrasting against the dark purple pyramids separated by a ribbon like haemorrhagic border zone Microscopically the changes appear as focal necrosis measuring a few millimetres and increasing up to complete necrosis of the cortex, leaving a thin sub-capsular cell layer

Since this fairly seldom occurring kidney disease was first described by *Friedländer* in 1883 (3), 71 cases were described until 1941 by *Duff* & *Moore* (1) and, according to a survey by *Sandritter*, dated 1967, 283 cases were recorded in 1961 (15) *Heptinstall* re commends more reticence in the registration of certain and well-defined cases (4)

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The majority of cases are seen in connection with pregnancy, prematurely terminated in the 5th to 8th month However, several cases of renal cortical necrosis associated with non obstetric diseases have also been observed It is known in men and has been observed in all age groups (2, 23, 24)

In view of the fairly small number of registered cases and also in order to draw attention to the connection with an aethiology other than the obstetric, the following cases observed by he authors are reported

MATERIAL AND METHODS

The period of registration was commenced in 1958 All six patients had been admitted to Rigs hospitalet. In all cases autopsy was made and tissue taken for microscopy During the same period (1958 1971 incl) a total of 5994 autopies were made During the period of registration no tissue from surviving patients with similar dis ease has been presented for examination Paraffin sections were made The registration of the microscopic details is based on a classification by *Sheehan* & *Moore* (17) a) few glomeruli affected the lesion being at a maximum of 0 5 mm (= focal), b) maximal lesion of 3 mm (= minor) c) ⅔ as

This work was supported by a grant from *The Danish Cystic Fibrosis Research Foundation*

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Fig 1 Total cortical necrosis (Patient no 1 × 1/2)

the interstitial tissue In two cases, fibrin like material was found in glomeruli (Fig 2) There was no exudate in the glomerular capsular space and glomerulus was not adherent to the capsule Minor arteries and arterioles in the infarcted areas showed sporadic fresh development of thrombi In one patient, (No 4) glomeruli with fibrosis were found in non affected areas—and both fresh and older thrombi were also in evidence in minor arteries and arterioles in areas which had no definite relation to the affected part

It appears that considerable changes were in all cases found in both kidneys—corresponding to the two most serious degrees according to Sheehan and Moore s graduation In one case (No 3) there were two sets of biopsy preparations and it was found that the lesion registered on the 29th day of the disease was somewhat less pronounced as com-

Fig 2 Necrosis of glomeruli with fibrin thrombosis in the glomerular capillaries (Patient no 2 ×100)

TABLE 2 *Other Autopsy Findings*

1	Uterus enlarged with coagulation remnants and diffuse myometritis Liver tissue with strong diffuse occurrence of acute inflammatory cells in the sinusoids Several suggillations
2	Uterus slightly enlarged with necrotic remnant of placenta *Minor subcapsular bleeding in* the liver, subendocardially on the septum cordis ecchymosis Lobar pneumonia.
3	Uterus with pronounced endo- and myometritis
4	Universal vasculitis with exclusively lymphocytic infiltration often necrotic walls of the vessels Skin with diffuse small petecchia and sugillations Pneumococcal sepsis Pulmonary *infarct and abscess and bilateral lobar pneumonia In the right half of the heart*, verrucous endocarditis with bacterial accumulations in fibrinous material Adrenal glands with vasculitis in surrounding fatty tissue and organized thrombosis in small arteries Bleeding in the adrenal gland medulla No changes of internal genitalia
5	Bilatral lung atelectases Chronic and acute cerebral anoxic changes
6	Icterus of skin and organs Many skin bleedings Bilateral bronchopneumonia Considerable sclerosis of the coronary arteries

pared with registrations on the 36th and 65th days of the disease when the changes were found to be of identical severity

Other essential autopsy findings appear from Table 2 It appears from the table that patient No 4 has lesions of other organs, thus implying collagenosis of the panarteritis nodosa type

DISCUSSION

The pathogenesis of bilateral cortical necrosis is grouped according to 1) obstetric complications and 2) other causes

In one of the present cases (patient No 4) the case history and various autopsy findings may imply a collagen complaint as also other kidney changes might imply a pre existing kidney complaint hence the findings cannot be unambiguously evaluated

In three patients (Nos 1 2 and 3) the disease developed in connection with pathologically terminated pregnancy This is in conformity with the well known fact that premature separation of placenta may—besides causing shock like conditions—give rise to sudden coagulopathy consisting i a in release of thromboplastin and thrombin in the blood with subsequent intravascular coagulation (19) In conformity with this fibrin like material was in one case (No 2) found in glomeruli (Fig 2) Other cases are observed in acute violent bleeding conditions In all these conditions, the presence of pre eclampsia or eclampsia (7, 12) is found simultaneously—but not necessarily Finally septic abortion is known as an aethiological possibility, possibly explained by influence of toxic substances of afferent arterioles and intralubular arteries (8) The present material comprised one patient of this category

In addition cases of bilateral cortical necrosis of clear non obstetric origin are described e g as a complication involved in various traumas shock stress etc (23)

Shock-like condition is known to be experimentally produced by intravenous administration of a few micrograms of endotoxin (complexes of polysaccharides polypeptides and lipidic derivatives of cell contents) from unspecified Gram negative bacteria, if repeated after 24 hours with the same or another endotoxin The reaction which is unspecific is easily induced in rabbits and does not give rise to changes after the first (conditioning) injection but produce the characteristic lesion 12 to 14 hours after the second (provoking injection) (8 22) This reaction in connection with experimental cholera was described by *Sanarelli* in 1924

(14) It is called the Sanarelli Shwartzman reaction or 'the generalized Shwartzman phenomenon' or just 'the Shwartzman phenomenon" according to his later description (18) of a lesion produced by intradermal injection of endotoxin in rabbits, after 24 hours followed by an intravenous endotoxin injection, causing intensive invasion of neutrophil granulocytes, leuco thrombocyte thrombi and necrosis of the vessel walls ('the local Shwartzman reaction') (6, 18) two to four hours later in the area of the first injection

The Shwartzman phenomenon produces microfibrin thrombosis of glomerular capillaries, afferent arterioles and intralubular arteries with ensuing renal cortical necrosis (22) The Shwartzman reaction is regarded as a coagulopathy with massive intravascular necrosis the kidneys being the site of predilection According to *Skjørten* (19), the microfibrin thrombi are considered the primary lesion in the intravascular coagulation The thrombi are present in the microcirculation in various organs but it is presumed that they are deposited in the glomerular capillaries and hence secondary thrombosis occurs in the area of the microfibrin lesion It is the secondarily extended changes which characterize the bilateral cortical necrosis and which are characteristic of the Shwartzman phenomenon (21)

In cases of simultaneous bilateral necrosis and *Esc coli* sepsis the possible connection between bilateral cortical necrosis and the Shwartzman phenomenon in man has been discussed The endotoxin formed by these infections probably causes the same provocation as the first endotoxin injection in the Shwartzman experiment (22) Bacterial endotoxin can reduce the time of coagulation and cause reduction of fibrinogen which, as far as time is concerned coincides with the occurrence of microthrombi Also other bacterial toxins may have this effect (11) A similar mechanism is reported in a bilateral cortical necrotic kidney lesion with simultaneous occurrence of Esc coli infection in children (10) Finally it has been suggested that a similar mechanism may be involved in conditions of hyperacute allograft rejection (13, 20)

Thus, it might seem as if there were two aethiologic possibilities of bilateral cortical necrosis, one purely obstetric and one non obstetric of multifactorial nature in which the mechanism in several cases may be considered the renal component of the generalized Shwartzman phenomenon Some obstetric cases can probably also be explained by this phenomenon, particularly cases due to septic abortion Furthermore, in abruptio placentae tissue, thromboplastin has been found in the blood simultaneously with reduced fibronogen and the occurrence of fibrin occlusion of a pulmonalis (16) In premature separation of placenta, post partal bleeding eclampsia and amnion liquid emboli afibrinogenaemia has been found (5) as it appears in the experimental Shwartzman phenomenon According to *McKay* (9), the vascular system in pregnant women is considered 'conditioned' corresponding to the first injection of the Shwartzman experiment, and the early separation of placenta releases substances in circulation from necrotic decidua, corresponding to the "provoking toxin administration in the Shwartzman reaction Furthermore, a modification by *Thomas* & *Good* (22) of the original Sanarelli experiment has shown that bilateral cortical necrotic lesions may develop after a single intravenous endotoxin dose in rabbits which either were pregnant or previously treated with steroids

The present material shows that bilateral cortical necrosis can present a widely varying clinical picture and apparently different pathogenesis On the basis of a brief survey of the literature concerning the generalized Shwartzman phenomenon, it is, however, considered justified to presume that these different case histories can be comprised by one common pathogenesis of this type

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Acta path microbiol scand Section A 80 357-366 1972

CELL SURFACE CHARGE AND METASTASIS FORMATION

A Study on the Effects of Dextrans and Heparin on Tumour Cells and Experimental Metastases in a Syngeneic Murine System

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An attempt was made to evaluate experimentally the importance of surface charge characteristics of tumour cells for growth of subcutaneous transplants and for metastasis distribution and growth in a syngeneic tumour host system (MCGI SS in CBA mice) The effect of nonsubstituted dextran (D) was compared to those of the polycation DEAE dextran (DEAE D) and the polyanion dextran sulphate (DS) Heparin was included in some of the experiments as an additional polyanion D, DS and heparin increased the net negative surface charge of the tumour cells while DEAE D drastically reduced or reversed it as determined by cell electrophoresis When given as intravenous pretreatment, the dextrans caused no significant changes in the total amount of metastases When added to the cell suspensions, dextran (D) and DEAE D increased the total metastasis crop by giving larger pulmonary metastases and increasing the number of tumour "takes" in other organs In the case of dextran (D), this was paralleled by a tendency to promoted growth of subcutaneously transplanted tumour cells, while DEAE D blocked the subcutaneous transplantability of the tumour cells This effect was reversible and could be removed by DS treatment of the cells DS treatment alone of the tumour cells did not affect metastasis formation to any significant extent This was in contrast to heparin treatment which seemed to shunt metastases past the lungs, i e the first capillary bed encountered after injection The lack of DS effect was tentatively explained as an interplay between impaired lodgement in vessels and promoted transplantability For DS treatment of cells improved their subcutaneous transplantability, evidenced by the quicker growth and larger resulting tumour volumes Heparin only hastened the growth of subcutaneously transplanted cells No evidence was found that the dextrans or heparin brought about the differences in transplantability or metastasis formation by altered aggregatability in the tumour cell suspensions Neither did they affect the viability index determined by dye exclusion of the tumour cells in suspension DEAE D seemed to increase the mechanical resistance of vigourously agitated cells in suspension, however

Surface properties of malignant tumour cells have been considered of decisive importance for their metastasis forming capacity (*Coman* 1953 1961, *Weiss* 1967) However, whether physico-chemical surface *characteristics* of tumour cells will determine the amount and pattern of metastases has not attracted much attention

Recently, a dispersing action of heparin on experimental, intravenously induced metastases has been interpreted rather in terms of direct cell effects than in terms of impaired blood coagulability (*Hagmar & Boeryd*

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1969a, b, *Hagmar & Norrby* 1970) Being a strong polyanion heparin may confer a higher negative surface charge onto the tumour cells, rendering them less liable to lodge in vessels. This hypothesis is supported by the findings that pretreatment of tumour cells gave a more pronounced effect than pretreatment of the animal recipients (*Hagmar & Norrby* 1970) and that other polyanions to some degree imitated the effect of heparin (*Hagmar & Norrby* 1970, *Suemasu & Ishikawa* 1970).

Consequently, it is of obvious interest to test how defined alterations of the surface charge of tumour cells will affect metastasis formation. Polyelectrolytes used for this purpose, e.g. for treatment of tumour cells before intravenous injection may, however, cause other disturbances which may hamper the interpretation of results. Thus apart from systemic effects, they may alter the aggregatability of cells in suspension (*Ambrose et al* 1958, *Sazl et al* 1962) and promote or impair their capacity for growth in the host (cf. *Takeuchi* 1966, *Lippman* 1968a, *Larsen & Thorling* 1969, *Thorling & Larsen* 1969).

In the present study attempts were made to evaluate these factors using tumour cells exposed to heparin and three types of dextran (MW 2×10^6): the polyanion dextran sulphate (DS), the polycation diethylaminoethyl dextran (DEAE D) and unsubstituted dextran (D). Changes in cellular surface charge densities were determined by electrophoresis. The state of aggregatability of the cells was tested *in vitro* and their viability by dye exclusion test and subcutaneous inoculation of a critically small cell dose (*Hagmar & Norrby* 1970). Experimental metastases were studied by intravenous infusion of tumour cells in media containing heparin or dextran. To evaluate the importance of systemic effects the dextrans in one experiment were instead given as intravenous pretreatment of the animals.

MATERIAL AND METHODS

The syngeneic sarcoma MCG1 SS in inbred CBA mice (*Mellgren et al* 1966) was used. The tumour cell suspensions were prepared with trypsin and DNase (*Boeryd et al* 1965). To obtain monodisperse suspensions with maximal viability the cells were spun down and resuspended in Parker 199 medium containing 10 per cent syngeneic mouse serum ($Parker_{90}$ $serum_{10}$) plus 0.6 mg per ml DNase (*Norrby et al* 1966). The cell counts were made in a haemacytometer, at least 40 fields ($1/160$ mm^3) being counted from each sample.

The dextrans were the commercially available Dextran 2000, DEAE-dextran and Dextran sulphate with 17 ± 0.5 per cent sulphate (AB Pharmacia, Uppsala, Sweden), all of mean molecular weight 2×10^6. Heparin without preservative (100 U/mg) was obtained from AB Vitrum, Stockholm, Sweden.

Cell Electrophoresis

The cylindrical tube apparatus described by *Bangham et al* (1958) was used. The determinations were made at $25 \pm 0.5°$ C and the field strength was 3.45 V/cm. Twenty cells were timed in each direction traversing a distance of 15 or 30 μM. Before and after each tumour experiment the apparatus was checked by running red blood cells in saline pH 7.2 (*Seaman & Heard* 1960). For reasons given by *Nordling* (1967) no attempt was made to calculate surface potentials. Instead the mobilities in μM/sec/V/cm were compared statistically by Students t test.

The measurements on tumour cells were always performed in $Parker_{95}$ $serum_5$ in order to have the same suspension medium as in the metastasis studies and to prevent the rapid devitalization otherwise to occur in simpler media (cf. *Haymouth* 1965). The electrophoretic determinations had to be performed at a cell concentration of 4×10^6 cells/ml to permit measurements on the rather quickly sedimenting tumour cells. Thus the concentrations of the test substances, assuming an absorption onto the tumour cells, would not be comparable to those in the less dense cell suspensions (see below!). Hence each substance was tested at two concentrations, one being identical in mg/ml with that in the metastasis experiments, the other being identical in mg/number of tumour cells (cf. *Mitchell & Cater* 1971). For the dextrans these concentrations were 1 mg/ml and 2 mg/10^6 cells (8 mg/ml), respectively. The viscosity of $Parker_{95}$ $serum_5$ was identical with water at 25° C determined according to *Hardwicke & Squire* (1952). For other solutions the mobilities were corrected for viscosity as described by *Mehrishi & Seaman* (1966). Each type of treatment was tested separately and compared to untreated cells run on the same occasion.

Aggregatability and Viability in vitro

Cell suspensions were diluted with Parker

serum$_{10}$ to approximately 10^6 cells per ml After assessing the accurate cell number by renewed counting 1 ml aliquots of the suspension were transferred to 10 ml sterile unsiliconized glass tubes containing 1 ml of Parker medium (control) or Parker-dextran medium (2 mg/ml of D, DS or DEAE-D) The sealed tubes were stored at 0° C or at room temperature and agitated either by gentle shaking every 15 minutes or in a vibrating agitator (Microid Flask Shaker Griffin & Tatlock Ltd., London) At the termination of an experiment samples were taken for the Trypan blue dye exclusion test and the suspensions were fixed by adding 4 per cent buffered formaldehyde to a final concentration of 0 4 per cent Such fixation preserves the degree of aggregation (Norrby et al 1966) A final cell count was made from the fixed suspensions The dilution induced with formalin was corrected for and differences in cell number were calculated in per cent

In the haemacytometer the attention was directed towards the presence of any cell aggregates This was also studied by observing in phase contrast the suspensions before and after fixation A drop from the suspension was applied to a slide in a 1 cm² square made of adhesive tape The small chamber was sealed by a cover slip

Subcutaneous Transplantability

One type of transplantation test (A) was performed with all 3 types of dextrans and with heparin Further studies (B) were made to clarify the effects of DEAE D

A Suspensions in Parker$_{95}$ serum$_5$ with dextrans (1 mg/ml), or heparin (0 2 mg/ml) or only medium (control) containing 10^3 MCGI SS cells per ml were stored on ice for 2 hours and gently shaken every 15 min One tenth ml of each suspension (10 cells) was injected subcutaneously into both groins of adult CBA mice Two consecutive studies were made with 6 and 8 mice for each type of suspension.

B 1) When Parker$_{95}$ serum$_5$ was made to contain 1 mg/ml DEAE D there was a lowering of pH from 8 0 to 7 5 DEAE D in Parker$_{95}$ serum$_5$ was therefore compared with two control suspensions one of pH 8 0 and the other one made pH 7 0 by addition of 0 1 N HCl Four animals in each group were used and 100 cells in 0 1 ml were injected into 4 subcutaneous sites Otherwise the study was performed as in A

2) In two consecutive studies the effect of storage time washing and neutralization of DEAE D were studied as well as systemic DEAE D treatment The mice received one tenth ml from suspensions in Parker$_{95}$ serum$_5$ with or without DEAE D containing 10^4 cells per ml (10^3 cells per dose)

The experimental groups were

I Controls, injected immediately with DEAE D free suspension

II DEAE D treated controls given 0 1 mg DEAE-D in 0 1 ml medium subcutaneously in the neck before injection of the DEAE D free tumour cell suspension

III DEAE D (1 mg/ml suspension), immediate injection

IV DEAE D (1 mg/ml suspension), 2 hours storage at 0° C before injection

V Washed DEAE D treated cells After gentle, manual agitation for two minutes in medium containing 1 mg/ml DEAE D, the cells were spun down (70 g 10 minutes), resuspended in Parker$_{95}$ serum$_5$ with adjustment of cell number and injected in the appropriate dose

VI DEAE D neutralization with DS A DEAE D containing (2 mg/ml) cell suspension (2 × 10^4 cells/ml) was stored on ice for 2 hours At that time and immediately before subcutaneous injection, the suspension was diluted to 10^4 cells by addition of the same volume of a 2 mg/ml DS solution

VII Controls given tumour cells stored for 2 hours at 0° C in DEAE D free medium.

In each of the two studies performed according to this model 6 mice were used in each group Groups VI and VII were included only in the last study, however and thus comprise a total of 6 mice each

The animals in all studies were examined daily for the development of palpaple tumours The tumour takes in each groin were recorded separately At the end of the observation period (21 days) the tumour bearing animals were killed and the tumours weighed In B 2) only the animals with bilateral tumours were killed which applies to all animals in Groups I, II VI and VII Animals in the other groups were observed for tumours appearing later The incidences of tumours were compared with chi square analysis and tumour weights with Student s t test

Metastasis Studies

Suspensions of MCGI SS cells in Parker$_{95}$ serum were injected into a tail vein Each animal received 0 1 ml of suspensions containing 5 × 10^5 cells/ml

In one type of study the dextrans (1 mg/ml) or heparin (0 2 mg/ml) were added to the tumour cell suspensions prior to injection One such study was performed in the 220th transplant generation of the tumour, using 12 animals in each group It was repeated in half scale in the 250th generation which study was performed simultaneously with the second type of experiment where the dextrans (0 1 mg in 0 1 ml) were given intravenously immediately before the injection of the tumour cells

The period of observation was 15 days The mice were sectioned and gross metastases noted The lungs and livers were prepared for histological examination according to *Boeryd* (1965). The total volume of metastases in per cent of organ tissue, the number of metastases per cm³ tissue and the mean metastasis volume in mm³ were planimetrically estimated from the sections (*Boeryd et al.* 1966).

Wilcoxon's two-sample rank test was used to compare differences between groups in numbers and volumes of lung and liver metastases Incidences of metastases were compared by chisquare analysis The mean numbers of gross extrapulmonary metastases were compared as described previously (*Hagmar & Boeryd* 1969b)

RESULTS

Cell Electrophoresis

The results are shown in Table 1 Significant, concentration-dependent increases of the electrophoretic mobilities were obtained with the two polyanions DS and heparin Of the two, DS gave a much more pronounced effect at the adopted dose-levels Dextran (D) also increased the electrophoretic mobility, although to a lesser degree than DS

TABLE 1 *Electrophoretic Mobility of MCG1-SS Cells in Parker$_{11}$-Serum, With or Without Dextrans or Heparin Mobilities Corrected for Viscosity at 25 ± 0 5° C*

Test substance	Conc mg/ml	Mobility μM/sec/V/cm (±SD)
Control cells		0 88 (±0 13)
Heparin	0 2	1 02ˢ (±0 15)
	1 6	1 16ˢ (±0 12)
Control cells		0 93 (±0 09)
Dextran (D)	1 0	1 10ˢ (±0 07)
	8 0	1 50ˢ (±0 09)
Control cells		0 86 (±0 09)
Dextran-sulphate (DS)	1 0	1 70ˢ (±0 15)
	8 0	4 02ˢ (±0 03)
Control cells		0 85 (±0 11)
DEAE dextran (DEAE-D)	1 0	0 47ˢ (±0 11)
	8 0	Isoelectrical

s = significant difference from control cells ($p < 0.001$)

DEAE-D reduced the mobility in a concentration of 1 mg/ml and when the concentration was raised to 8 mg/ml (2 mg/10⁶ cells), the cells lost their mobility, i e became isoelectrical at the plane of shear. Occasional cells were observed to move towards the cathode, i e. on these cells there was a reversal of the surface potential

Aggregatability and Viability in vitro

Regardless of type of agitation used or whether the cell suspensions were stored at 0° C or at room temperature, no evidence for aggregation was found in the truly monodisperse suspensions There was a fall in cell number in all suspensions during storage, probably by cell lysis, but without differences between the different types of suspension The results of two experiments with gentle manual agitation (inverting sealed tubes 5 times every 15 minutes) are shown in Table 2 The table also shows that the viability index, assessed with Trypan blue, was similar in all types of suspension No cell aggregates were observed in the haemacytometer or in the slide chamber, regardless of the type of suspension

When the suspensions were vigorously agitated in the vibrating shaker a curious difference between the DEAE D and the other types of suspension was repeatedly demonstrated While there still was no aggregate formation, the cell loss was significantly less in the DEAE-D suspension than in any of the other suspensions (*Table 3*) Variations in pH of control suspensions did nothing to alter the difference

Subcutaneous Transplantability

A The results were similar in the two consecutive tests and the combined results are plotted in Fig 1 The tumour development began earlier from the heparin and DS-containing suspensions The DS-suspensions also gave a higher incidence of "takes" and greater tumour weights than the control suspensions ($p < 0.01$) The tumour weights were not significantly different from the con-

TABLE 2 *Cell Number and Viability Index in MGCI-SS Suspensions Stored for 2 Hours under Gentle Manual Agitation*
Viability Index – Number of Trypan Blue Unstained Cells/Total Number of Cells

Medium Parker$_{85}$ serum supplemented with	Cell number per ml ($\times 10^3$) after storage (Viability index within brackets) 0° C Initial cell number 600×10^3 cells/ml	 Room temperature Initial cell number 518×10^3 cells/ml
1 – – – (control)	577 (0 95)	484 (0 93)
2 Dextran (D) 1 mg/ml	542 (0 89)	488 (0 94)
3 D sulphate 1 mg/ml	573 (0 96)	504 (0 97)
4 DEAE D 1 mg/ml	577 (1 00)	512 (0 98)

trols in the D (dextran) or heparin groups $(p > 0\ 10)$ The incidence of takes, however, tended to rise in both these groups $(p < 0\ 10)$ Only one tumour developed from the DEAE D suspension

B 1) The two control suspensions (pH 7 0 and 8 0) gave rise to similar numbers of takes, 11/16 and 8/16, respectively, and similar tumour weights 0 58 (± 0 22) and 0 52 (± 0 43) g The DEAE D suspension only gave rise to one small tumour (0 09 g)

2) Fig 2 shows the effect of storage in DEAE D and how washing off or neutralization of DEAE D with DS affected the transplantability of the tumour cells The transplantability was much impaired when the cells were injected in DEAE D containing medium whether immediately injected (Group III) or stored for 2 hours prior to injection (Group IV) The transplantability could partly be restored by washing (Group V) A complete restoration of transplantability was achived by neutralization with DS, even after 2 hours exposure to DEAE D (Group VI) Systemic DEAE D treatment did nothing to impair the transplantability (Group II) No significant differences in tumours weights were found in the simultaneously killed groups (I, II, VI and VII) One tumour developed in each of Groups III, IV and V after day 21 (not shown in Fig 2)

Metastasis Studies

As can be seen in Table 5 there were only slight differences in the amount and distribution of metastases when the dextrans were given as intravenous pretreatment The only significant changes were an increased number of lung metastases in Group D and a reduction in mean volume of liver metastases

TABLE 3 *Cell Loss During Vigorous Mechanical Agitation at Room Temperature*
Viability Index – Number of Trypan Blue Unstained Cells/Total Number of Cells

Medium Parker$_{85}$ serum, supplemented with	Cell loss in per cent of original cell number (Viability index within brackets where tested) 1 5 h agitation Initial cell number 712×10^3 cells/ml	 2 h agitation Initial cell number 644×10^3 cells/ml
1 – – (control)	47 (0 91)	79
2 Dextran (D) 1 mg/ml	50 (0 98)	77
3 D sulphate 1 mg/ml	53 (0 93)	72
4 DEAE D 1 mg/ml	8 (0 99)	40

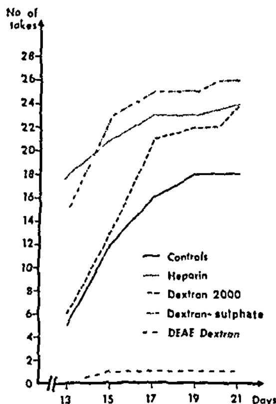


Fig 1 Number of tumour takes developing after subcutaneous transplantation of 10^2 MCG1 SS cells stored for 2 h at 0° C in medium containing heparin (0 2 mg/ml) or dextrans (1 mg/ml) 14 animals in each group were injected bilaterally Mean tumour weights (± SD) in grams were controls 0 56 (± 0 50) heparin 0 80 (± 0 56) dextran 2000 0 66 (± 0 52) dextran sulphate 1 03 (± 0 57) and DEAE dextran 0 20 ( )

in Group DEAE-D These changes did not alter the total volumes of metastases in the respective organs, however, not even at the 10 per cent level

Nor when given in the cell suspension (Table 4) did DS change the metastasis parameters significantly Heparin in the cell suspension increased the number of gross extrapulmonary tumour takes, but other changes were below the level of significance In contrast, dextran (D) and DEAE D caused increased metastasis volumes in the lungs where DEAE D also tended to increase the number of metastases, similarly as in the liver ($p < 0\ 10$) In addition the number of gross extrapulmonary tumour takes rose in the D and DEAE D groups Similar trends were obtained in the repeated half scale experiment (not shown in the table) D, DEAE D again increased the total metastasis crop by augmenting the number of gross extrapulmonary tumour takes, while other changes did not attain significance

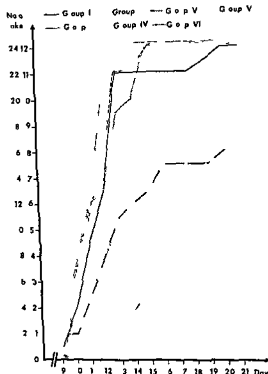
DISCUSSION

In the present study metastasis formation was enhanced when tumour cells were infused in medium containing unsubstituted dextran (D) and DEAE D No comparable increase was obtained when the same dose

TABLE 4 *Metastases in Mice Given 5 × 10⁴ MCG1-SS Cells Intravenously Heparin (0 2 mg/ml) or Dextrans (1 mg/ml) Added to Cells Suspended in Parker₀₅ Serum*

Group	To lungs				To liver				To other organs	
	Incidence	Percentage tumour in organ	Mean volume mm^3	Mean number per cm^3	Incidence	Percentage tumour in organ	Mean volume mm^3	Mean number per cm	Incidence	Mean no
Controls	12/12	2 8	0 33	85	9/12	3 1	1 2	25	6/12	1
Heparin	12/12	0 9	0 09	94	8/12	2 6	2 9	9	10/12	[illegible]
Dextran (D)	10/12	4 7s	0 51	91	9/12	6 9	1 8	39	10 12	[illegible]
D sulphate	12/12	2 3	0 37	64	10/12	6 7	1 7	39	9/12	1
DEAE D	12/12	6 3s	0 69	91	11/12	7 8s	1 9	41	10/12	3

s – significant difference from controls ($p < 0\ 05$)



F g 2 Number of tumour takes de elop ng after subcutaneous transplantat on of 10³ MCGI SS cells exposed or unexposed to DEAE dextran The groups (more fully described on page 3) were
Group I untreated immed ately njected cells
Group II I but m ce pretreated w h 0 1 mg DEAE D
Group III
Group IV III but cells stored for 2 h *in vitro*
Group V DEAE D treated cells washed prior to nject on
Group VI cells stored for 2 h in DEAE D me d um neutral zed before inject on w th dextran sulphate
Group VII untreated cells stored for 2 h *in vitro* prior to inject on
In Groups I V 12 m ce were injected b laterally wh ch gave a max mum of 24 humour takes (left column at the vert cal axis) Groups VI and VII comprise only 6 mice each and hence max mally 12 takes (right column)

of the dextrans was given as intravenous pre treatment Although the total metastasis crop was somewhat different in the two experi ments the discrepancy ind cates that meta stas s formation was promoted rather by alteration of the tumour cells than by systemic effects

Dextran (D) also tended to increase the takes frequency of subcutaneously inoculated tumour cells Whether due to interaction with immunological host defence mechanisms or to a more unspecific protection of the cells such facilitated transplantabil ty may explain the metastasis results at least in part Such interpretations l ave been applied to analog ous results with low molecular weight dextran (*Hagmar* 1971) The results may also be compared to previously demonstrated gener

TABLE 5 *Meta tases in M ce G ven 5 × 10⁴ MCGI-SS Cells Int avenously The D fferent Types of Dextran (0 1 mg n 0 1ml) Were G ven Intravenously Immed ately Before the Tumour Cell Inject on*

Group	To lungs				To l er				To other organs	
	I c dence	Percentage t mour n organ	Mean volume mm³	Mean number per cm³	Inc dence	Percentage tumour n organ	Mean volume mm³	Mean number per cm³	Inc dence	Mean no per n m
Controls	11/12	6 3	0 91	68	7/12	1 8	2 90	6	11/12	6 1
Dextran (D	11/12	7 2	0 43	170ˢ	8/12	2 4	1 84	9	11/12	5 7
D sulpha e	12/12	6 4	0 48	134	7/12	2 4	2 70	15	12/12	3 8
DEAE D	12/12	5 1	0 45	114	9/12	1 6	1 10ˢ	13	12/12	4 6

ˢ s gn f cant d fference from controls ($p < 0.05$)

ally promoting effects of dextran on experimental metastases (Survey of Literature, *Hagmar* 1971).

In the case of DEAE-D, effects by enhanced transplantability can be excluded since DEAE-D blocked the subcutaneous *transplantability of the tumour*. That this was not due to killing of the tumour cells prior to injection is borne out by an unaltered viability index during storage and, more convincingly, by reversability by DS neutralization of DEAE-D after 2 hours' exposure *in vitro*. The effect was also independent of the exposure time. These findings amply confirm the concept advanced by *Larsen & Olsen* (1968) and *Larsen & Thorling* (1969) *that DEAE-D induces a reversible transplantation inhibition*, perhaps by altering the tumour-host relationship (*Thorling et al.* 1971). The DEAE-D effect, which is presumed to take place by alteration of the tumour cell surface, was less pronounced when the cells were washed prior to injection. This and the DS-effect presumably reflects the situation when the tumour cells are intravenously transfused when DEAE-D will be diluted in blood and at least in part neutralized by anionic plasma proteins. A remaining DEAE-D effect seems in this situation to have promoted the lodgement of cells in organs where they were able to proliferate into a greater number of metastases than in the controls. The nature of this effect is as yet unknown. No evidence was found that DEAE-D caused tumour cell aggregation prior to injection which otherwise might have facilitated their retention in organs. DEAE-D increased, however, the mechanical resistance of the tumour cells as evidenced by the agitation test. This effect, the nature of which is yet unexplored, may have helped tumour cells to resist mechanical injury in the blood stream. Another reasonable assumption is that the DEAE-D treated cells with a reduced or reversed surface charge will be subjected to less repulsion from the endothelium with greater facilities to lodge in vessels and to penetrate the vascular wall (cf. *Weiss* 1967).

A reverse situation would be expected for DS and heparin treated cells which at the moment of injection had an increased negative surface charge. This obviously takes *place* by adhesion of the polyanions onto the cell periphery, as demonstrated morphologically for heparin in this system (*Hagmar & Norrby* 1970). Heparin reduced, although not significantly, the amount of lung metastases, while *it increased the number of takes in other* organs. This may, like previously (*Hagmar & Boeryd* 1969a, b), be interpreted in terms of a shunting of tumour cells past the first capillary bed, the lungs. More cells would then be available for growth in other organs. Here the growth also may be facilitated by *the heparin treatment. The subcutaneously* transplanted heparin treated cells grew faster than the control cells. The resulting tumour volumes did not differ significantly, however, as in a previous study (*Hagmar & Norrby* 1970). The quicker outgrowth may be compared to the results obtained by *Lippman* (1968a), according to whom a promoted transplantability may be interpreted immunologically and discrete from a growth restraining effect *in vitro* (*Lippman* 1968b).

Dextran sulphate (DS) increased the surface charge of the tumour cells more than heparin and had a more pronounced growth promoting effect on subcutaneously transplanted tumour cells. If a similar trans*plantation promoting effect* prevails also when the tumour cells are given by the intravenous route, it may have masked an inhibited lodgement in vessel, explaining the unaltered metastasis crop. Dextran (D) treatment of the cells also increased their net surface charge, however, probably by covering cationic groups or otherwise changing surface proteins (*Ross & Ebert* 1959). This change did not impair the lodgement of cells in vessels as evidenced by increased metastasis volumes even in the lungs. These findings tend to minimize the role of surface charge as a determinant of metastasis development from intravenously injected cells.

Thus the present results do not provide the basis for any precise evaluation of various

suggested mechanisms by which the dextrans may alter metastasis formation The study gives some additional support, however, to the hypothesis that physicochemical characteristics of the cell periphery can be important in determining the fate of tumour cells released into the blood Parallels may be drawn to the altered distribution of lymphocytes subjected to surface charge reducing enzyme treatment (*Gesner & Ginsburg* 1964, *Woodruff & Gesner* 1969) and to the different distribution patterns obtained with anionic and cationic latex particles (*Wilkins* 1967) These results suggest that electrical charge characteristics may be one of the factors determining the distribution of injected cells or particles As already pointed out, treatment affecting this property is likely to achieve complex interactions with cell functions, however, as well as with the relationship to the host It is to be hoped that further studies of alterations, as selective as possible, of tumour cell surfaces will help to elucidate the relative importance of the mechanisms involved

The study was supported by research grants from the *Swedish Cancer Society*

The skilfull technical assistance of Miss *Marianne Bremner* is gratefully acknowledged

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Acta path microbiol scand Section A 80 367–373, 1972

STUDIES ON THE *IN VIVO* EFFECT OF POKE WEED MITOGEN (PWM) ON THE LYMPHOID TISSUE OF THE RAT

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Intraperitoneal injections of PWM in rats had no effect on the haemoglobin concentration or leucocyte and lymphocyte counts A transformation of the lymphocytes of the peripheral blood to large 'blast like' DNA synthesizing cells – as seen *in vitro* – did not occur PWM produced a significant increase of the spleen weight and the spleen follicles and the red pulp showed an increased cellularity with many pyroninophilic large immature lymphocytes The lymph nodes showed disorganization of the normal structure with an increased number of immature lymphocytes, reticulum cells and plasma cells The bone marrow and thymus were unaffected

The *in vitro* effect of poke weed mitogen (PWM) and phytohaemagglutinin (PHA) on human lymphoid tissue has been well-established, whereas studies on their *in vivo* effect in man and mammals are scarce and have given inconsistent results Thus a transformation of the peripheral blood lymphocytes to large "blast-like' cells has been observed in children after systemic exposure to PWM (*Barker et al* 1966) PHA has been used in the treatment of aplastic anaemia with doubtful results (*Humble* 1954, *Fleming* 1964, *Retief et al* 1964) Both agents have been given to rodents, and morphological changes have been observed in the peripheral blood lymphocytes and/or spleen and lymph nodes by some authors (*Gamble* 1966 *Haase et al* 1968, *Lozzio et al* 1969, *Zazula & Fikrig* 1970, *Hartveit* 1970) whereas others were not able to demonstrate any effect (*Elves et al* 1963) The aim of the present study has been to ascertain whether PWM exerts an effect on the peripheral blood lymphocytes, bone marrow, thymus, spleen and lymph nodes of the rat

MATERIAL AND METHODS

Four week-old female Wistar rats were used PWM (Grand Island Biological Company) was reconstituted with sterile water and used immediately Three groups of animals (three in each) were given 1, 10 and 100 mg PWM per kg body weight intraperitoneally, respectively On days 0, 1, 2, 3, 5, 7, 10, 14, 21 and 42, smears were prepared from peripheral blood taken from the tail vein and 200 cells counted Leucocyte counts were carried out on days 0, 1, 2, 3, 4, 5, 7, 10 and 14, and haemoglobin concentration on days 0, 3, 5 and 7 in the rats which had received 1 and 10 mg PWM per kg body weight In five control animals, haemo-

Received 27 ix 71

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globin concentration and leucocyte counts were carried out on days 0, 3, 5 and 7.

Two groups of animals (six in each) received 1 and 10 mg PWM per kg body weight intraperitoneally, respectively. On days 5 and 10 three ml of blood was obtained by cardiac puncture from three rats of each group. A control group of three rats did not receive PWM, but was otherwise treated as described below. One µCi of ³H thymidine (1.9 Ci/Mmol, Schwarz Bio Research, Inc.) was added to each specimen which was incubated for one hour at 37° C. Blood smears were made and the slides were fixed in absolute methanol and processed for autoradiography with Kodak NTB-2 emulsion. After exposure for one week, the slides were developed and stained with Giemsa at pH 5.75. Five hundred cells were counted from each rat.

Ten rats received 10 mg PWM per kg body weight intraperitoneally. Five and 11 days later five rats were sacrificed. A lymph node from the axillary region, the thymus and the spleen were removed and fixed in formalin for histological study. Sections were stained with haematoxylin and eosin (H-E), methylgreen pyronine, toluidine blue and Giemsa. Six control rats which did not receive PWM were treated in the same way.

TABLE 1. *Proportion of DNA-Synthesizing Mononuclear Cells in Peripheral Blood From Rats Treated With Pokeweed Mitogen (PWM)*

Dose of PWM mg per kg body weight	Days after treatment	No of labelled cells/500 cells
1	5	3
1	5	0
1	5	0
10	5	0
10	5	2
10	5	2
1	10	4
1	10	1
1	10	6
10	10	5
10	10	6
10	10	7
0	10	0
0	10	3
0	10	2

RESULTS

1 Effect on the Haemoglobin Concentration and Leucocyte Count

PWM had no effect on these parameters. Nor were there any changes in the leucocyte count.

2 Effect on the Morphology of the Lymphocytes of the Peripheral Blood

No changes in lymphocyte morphology were seen. Thus a transformation of the lymphocytes to large "blast-like" cells capable of synthesizing DNA—as seen in *in vitro* cultures of the peripheral blood—was not found in any of the rats treated with PWM. Table 1 shows the percentage of labelled lymphocytes 5 and 10 days after the administration of PWM. It is seen that the proportion of cells in DNA synthesis did not exceed the control values.

3 Effect on Bone Marrow Cells

No quantitative nor morphological changes were seen within the various cell types of the marrow.

TABLE 2. *Relative Spleen Weight* in Rats Treated With Pokeweed Mitogen (PWM) (Values Are Means ± SE of the Mean)*

Group	No of rats	Dose of PWM mg per kg body weight	Days after treatment	Spleen weight in % of body weight
Control	6	0	11	0.312±0.012
PWM	5	10	5	0.440±0.015§
PWM	5	10	11	0.360±0.019

§ Difference between control and this experimental group is significant ($p < 0.001$)

* Spleen weight in per cent of body weight

4 *Effect on the Spleen*

Table 2 shows the relative spleen weight (spleen weight in per cent of body weight) of the rats treated with PWM and the controls There was a statistically significant increase of the relative spleen weight five days after the administration of PWM

Histologically, the spleen 5 days after treatment compared with the controls showed a varying enlargement of the follicles (Figs 1 and 2) These follicles demonstrated increased cellularity and germinal centres with many pyroninophilic cells On the whole there was a striking increase in the cell density of the red pulp A great number of pyroninophilic large lymphocytes or lymphoblast like cells had appeared together with scattered small lymphocytes (Fig 3) The number of megacaryocytes varied considerably in each individual and no significant morphological changes were observed Eleven days after administration of PWM the splenic changes showed evident regression However, enlarged germinal centres were still seen and the red pulp still showed a slightly increased cellularity with nucleated pyroninophilic cells of the same kind as after 5 days (Fig 4)

5 *Effect on the Lymph Nodes*

Five days after the injection the normal structure showed moderate disorganization with marked proliferation of the follicles and larger confluent ' follicle like elements with prominent germinal centres In most of the lymph nodes a moderate oedema could be seen and the dilated sinusoids contained a great number of small lymphocytes Furthermore, pyroninophilic cells were seen in the sinusoids as well as in the paracortial and medullary areas Several small necroses were found paracortically, and in the germinal centres and many of them contained necrotic lymphocytes The endothelial cells lining the sinusoids and capillaries were often swollen and proliferating Eleven days after PWM treatment the disorganization of the normal structure was still present and in most cases to an even greater extent than after 5 days (Fig 5). The prominent germinal centres showed a marked pyroninophilia. Paracortically, immature reticulum cells with bright, slightly enlarged nuclei were seen together with plasma cells and large lymphoblast-like cells showing pyroninophilia Especially in the hilus region a great number of these cells were present (Fig 6) Regarding the number and morphology of mast cells, no significant changes could be observed, although it appeared as if an increasing number were found after 5 and 11 days compared with the controls The degree of degranulation showed considerable variation

6 *Effect on the Thymus*

No significant changes were seen in the thymus compared with controls There seemed to be no changes in the cell population and although the depth of the cortex was subject to slight variation, a so called "cortial depletion" was never observed

DISCUSSION

The present studies demonstrate that PWM has no effect *in vivo* on the haemoglobin concentration or leucocyte count in the rat From a morphological as well as from an autoradiographic point of view, there was no increase of transformed DNA synthesizing cells in the peripheral blood of the rats treated with PWM In this context it may be mentioned that peripheral blood lymphocytes of the rat are responsive to PWM *in vitro* (*Stayner* & *Schwarz* 1969) PWM produced an increase of the spleen weights of the rats, and morphological changes were seen in the spleen and lymph nodes, whereas the morphology of the thymus and bone marrow seemed unaffected

Histological findings similar to those described above have previously been reported in mice and rats treated with PHA and PWM (*Gamble* 1966 *Zazula* & *Fikrig* 1970 *Hartzell* 1970) The abnormal thymus histology of mice treated with PHA described by *Hartzell* (1970) could not be reproduced in the rats treated with PWM This might pos-

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Fig 1 Normal spleen from control rat H-E × 84

Fig 2 Spleen from rat 5 days after one intraperitoneal injection of PWM Note enlargement of follicles and increased cellularity of the red pulp H E × 84

Fig 3 Enlargement of fig 2 Red pulp of spleen with a great number of lymphoid cells H-E × 525

Fig 4 Spleen from rat 11 days after injection of PWM Enlargement of follicles is still present Almost normal cellularity of the red pulp H E × 84

Fig 5 Lymph node of rat 11 days after injection of PWM Disorganization of the normal structure and prominent germinal centres H-E × 210

Fig 6 As fig 5 Collections of immature pyroninophilic lymphoblast like cells and *plasma* cells H E × 525

sibly be due to the fact that different mitogens were employed in the two studies Recently, some evidence has been presented that PHA acts on thymus-dependent lymphoid cells whereas PWM stimulates lymphoid cells of the non thymus-dependent system (*Stockman et al* 1971)

The significance of the histological alterations produced *in vivo* in the lymphoid tissue of the rat is unknown The most distinct finding, viz an increased number of pyroninophilic large immature lymphoid cells may be the equivalent of the transformation of lymphocytes in *in vitro* cultures

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This work has been supported by a grant from *Anders Hasselbalch's Fond til Leukæmiens Bekæmpelse*

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Acta path microbiol scand Section A 60 374-378, 1972

ABNORMAL MORPHOLOGY OF GERM CELLS IN TWO INFERTILE MEN

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An abnormal morphology of germ cells has been found in two men with severely reduced fertility Generally, in addition to Sertoli cells, only one cell type was present in the seminiferous tubules Cells of this type differed considerably from normal spermatogonia The diameter of the nuclei was considerably increased and they stained intensely with Feulgen stain Spermatocytes and spermatids were absent in the abnormal tubules Few apparently normal, tubules were also present, which accounts for the finding of a small number of spermatozoa in the semen specimens Both urinary excretion of gonadotrophins and 17 ketosteroids as well as secondary sex characteristics and chromosome complements were normal

In the majority of men with oligospermia and reduced fertility, a reduction in the number of germ cells is present The morphological characteristics of the spermatogonia spermatocytes and spermatids are generally not different from those of the germ cells in normal men, except that degenerated cells are frequently present (*Nelson* (7)) However, in a study of men with severely reduced fertility, two unrelated men were found to present the same abnormality in the morphology of the germ cells The aim of the present communication is to describe the abnormal testicular cytology and to report some endocrinological and clinical data obtained from an investigation of these patients who apparently are the first of their kind to be reported

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MATERIALS

Case 1

A 30 year-old writer presented himself at an infertility clinic after two years of barren marriage There was no history of undescended testes and puberty developed normally He had never had any disease of the genitalia or other major somatic diseases but had been treated at an out patient clinic for neurosis His sex life was normal

Physical examination Height 191 cm weight 75 5 kg Secondary sex characteristics normal. Right testis 12 cm³ and left testis 18 cm³ measured by orchidometer (Normal range 15-35 cm³) Both testes located in scrotum No varicocele Prostate moderately enlarged with normal consistency and not tender

Laboratory data The results of sperm analysis are shown in Table 1 The urinary excretion of total gonadotrophins was 18 MUU/24 h (normal limits 6 75 MUU/24 h) Excretion of total 17 ketosteroids was normal Analysis of X-chromatin was negative and he had a normal 46,XY chromosome complement of lymphocytes

Case 2

A 29 year old economist with a history of 3 years of barren marriage During childhood he was

TABLE 1 *Sperm Analyses*

Case	Volume cm³	Sperm mill/cm³	Abnormal heads %	Mobile sperm %	Motility
1	4	2 8	7	76 (¾)	Fair
	4	2 0	20	73 (1¼)	Quite good
2	1 6	0 09	*	40 (¾)	Poor
	1 4	0 3	*	75 (½)	Poor

( ) Hours after ejaculation
* No differential count performed

treated in hospital for neurosis Left testis was located in scrotum at birth and right testis descended after a series of choriongonadotrophin injections when the patient was 9 years old He had an operation for lateral hernia on the right side at the age of 13 He developed normally at puberty Libido and potentia were normal

Physical examination Height 184 cm weight 65.5 kg Secondary sex characteristics normal Right testis 16 cm³, left testis 27 cm³, measured by orchidometer Both testes are located in scrotum No varicocele Prostate normal

Laboratory data The results of sperm analysis are given in Table 1 Measurement of urinary excretion of total gonadotrophins showed 7 and 18 MUU/24 h at two occations (normal limits 6 75 MUU/24 h) The excretion of total 17 ketosteroids in the urine was normal The analysis of X chromatin was negative and the chromosome analysis of lymphocytes showed a normal 46,XY karyotype

METHODS

Material from the right testis was removed surgically during local anaesthesia The biopsy from Case 1 was fixed in Stieve's fixative and embedded in paraffin, while the material from Case 2 was fixed in Cleland s fixative and embedded in paraplast. Biopsies were sectioned at 4 μ and stained with iron haematoxylin according to a reported method (*Rowley et al* (10)) Furthermore one slide from Case 2 was processed by the Feulgen staining method The sperm analyses were performed by the method of *Hammen* (3) The urinary excretion of gonadotrophins were measured by the methods of *Johnsen* (5, 6)

RESULTS

All tubules in the biopsy of Case 1 showed the same abnormality In Case 2 the same abnormality was found in 95 per cent of the tubules while the rest was normal (Fig 1a) In addition to Sertoli cells abnormal tubules generally contained only one cell type with the following characteristics The cells were located in one layer along the tubular wall with a broad contact between an abundant



Fig 1 a section showing abnormal tubules (A) located together with a normal tubule (N), × 100, b high magnification of an abnormal tubule showing one layer of abnormal cells (G) along the tubular wall and a series of Sertoli cells (S) with normal appearance, × 400 Iron haematoxylin stain

cytoplasm and the tubular membrane (Fig 1b) although a few cells were seen closer to the lumen The nuclei were almost spherical and contained one to four large irregular chromatin clumps located in the centre of the nucleus as well as smaller chromatin masses Only very little chromatin was attached to the nuclear membrane The size of the nuclei was significantly increased in comparison with normal spermatogonia (Table 2) and estimated from the Feulgen stained slides the DNA content was much higher than in normal spermatogonia (Fig 2) The Sertoli cells in these tubules were located somewhat further from the tubular membrane than in normal tubules as if the layer of the large abnormal cells had moved the Sertoli cell nuclei towards the centre of the tubule (Fig 1b) A differential count was performed on Sertoli cells and abnormal cells in 15 of the abnormal tubules in Case 2 The ratio was approximately 1 1 An occasional spermatogonia with normal appearance was observed in some of the otherwise abnormal tubules Several mitotic divisions were seen (Fig 3) in these abnormal tubules while meiotic divisions were never observed Measurement of 10 cross sections of abnormal tubules in each case showed that the tubular diameter was decreased In Case 1 the tubular diameter varied between 89 162 μ with an average of 107 μ (normal range in this laboratory 197 $\pm$ 30 μ) The diameter of the abnormal tubules in Case 2 varied between 106 and 146 μ and the average diameter was 128 μ The diameter of the few tubules with normal spermatogenesis was normal The thickness of the tubular wall was increased uniformly though only to a minor degree This apparent increase may be due to shrinkage in tubular diameter and length No tubules were totally obliterated in either of the cases Except for the change in location towards the centre of the tubules no change could be revealed by microscopy of the Sertoli cells



Fig 2 Feulgen stained section with a normal and abnormal tubule Note the heavy uptake of stain by the abnormal cells (X) compared with the normal spermatogonia (N) Also note the moderately stained Sertoli cells (S) × 500

The Leydig cells appeared to be normal in

TABLE 2 *Average Nuclear Diameter of the Atypical Germ Cells in Cases 1 and 2 Compared with Average Diameter of Nuclei of Normal Spermatogonia*

Atypical germ cells				Normal spermatogonia			
Case 1		Case 2		Stieve's fixative		Cleland's fixative	
Mean μ	S D	Mean μ	S D	Mean μ	S D	Mean μ	S D
10 4	0 9	10 0	0 9	6 9	0 6	6 5	1 0

Counts of 20 randomly chosen nuclei in each case
S D — Standard deviation

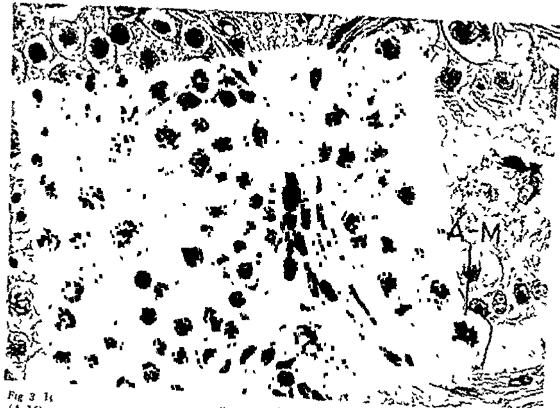


Fig 3 I[illegible] (A-M) [illegible] n a normal and an abnormal tubule Note mitotic division Iron haem[illegible] [illegible]ium stain [illegible]uble similar to the mitotic division (N-M) in the *normal* tubule, ×650

both cases but these were not quantitated No Leydig cell nodules were seen

DISCUSSION

The identity of the unusual cell type found in the seminiferous tubules of these two men is not clear However, it must be presumed that the cells are derived from germ cells, since divisions were seen and normal numbers of Sertoli cells with normal appearance were found in all tubules Furthermore, the abnormal cells were not similar to the normal or abnormal Sertoli cells found in testicular failure (*Skakkebæk* (11) *Fround et al* (2)) The location of the cells in one layer close to the tubular wall suggests that they may be abnormal spermatogonia although in many respects the morphology was different from that of the spermatogonia of normal men (*Clermont* (1)) Of the normal spermatogonia, Clermont s type B has most in common with the cell type under discussion However, the chromatin in type B is finer and, normally, only one to two large chromatin masses are located in the centre of the nucleus Furthermore type B spermatogonia are usually not so firmly attached to the tubular wall (*Clermont* (1) *Rowley et al* (9)) Also the size was different from normal spermatogonia Compared with normal spermatogonia fixed by the same methods, the nuclear diameter of the abnormal cells was in both patients increased by approximately 50 per cent

Apart from the size there were few similarities to normal spermatocytes No organization of the chromatin threads was visible, although the amount of DNA judged from the Feulgen stained sections appeared to be

substantially increased, as is the case in primary spermatocytes following the DNA synthesis in preleptotene spermatocytes (*Heller et al.* (4)).

The fact that tubules containing normal germ cells were found side by side with abnormal tubules suggests that the cause of the abnormal cell development may not be found in the interstitial tissue. Furthermore, the Leydig cells appeared to be normal both in number and morphology.

Neither is it likely that abnormalities in the production and plasma level of gonadotrophins were the causal factor. Urinary excretion of 'total gonadotrophins' was normal and the existence of apparently completely normal spermatogenesis in a few tubules also indicates that normal gonadotrophin stimulation was present.

The thickness of the tubular walls of the abnormal tubules was somewhat increased and it is possible that the passage of hormones and nutritional factors may have been hampered. However similar changes of the tubular wall are often found in subfertile men without any association with morphological changes of the germ cells demonstrable by light microscopy.

It would appear that the most likely causal factor is to be found in the cells themselves or in the Sertoli cells. The Sertoli cells although apparently normal by light microscopy may be functioning inadequately. Unfortunately little is known about the role of the Sertoli cell in the human spermatogenesis (*Paulsen* (8)).

I am grateful to professor *G. Teilum* for valuable discussions, *J. Philip M.D.* for chromosome analyses, *H. Rebbe M.D.* for performing testicular biopsies, *Hans Lyon* M.D. for the Feulgen staining of a slide and Mrs. *Annelise Persson* for technical assistance. This work was supported by the P. Carl Petersen's Fund.

ADDENDUM

Since this paper was prepared I have come to know of a patient with the identical type of abnormal germ cells (Paulsen and de Kretser, personal communication). In their patient the abnormal type was more common on one side and Serum FSH, Serum LH and chromosome complement were normal.

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Acta path microbiol scand Section A 80 379-390 1972

RENAL TRANSPLANTATION IN RABBITS

VI Immunofluorescent Study of IgG Deposits in Renal Allografts

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IgG deposits in 74 renal allografts from non related rabbits were studied by direct immuno fluorescence technique. The allografts were removed 1 to 14 days after transplantation Vascular deposits of IgG were demonstrated from the second day and glomerular IgG deposits from the fourth day after transplantation The frequency of IgG deposition in intrarenal blood vessels (13 cases) and in glomeruli (6 cases) was low, however, and no correlation was found between time and localization of IgG deposits and light microscopic alterations even not in the more severe allograft reactions

Light microscopic examination of renal allo grafts from unrelated rabbits have shown qualitative and quantitative variations in the histological pattern when compared at the same time after transplantation (*Lund & Myhre Jensen* III, 1970) Several observa tions among them also the demonstration of kidney specific antibodies in venous blood from dog allografts obtained 2 days after transplantation (*Spong et al* 1968) makes it possible that humoral antibodies play a role in the acute rejection of the allograft beside the cellular type of response and that it is important for the severity of the allograft reaction

In the present study we have examined IgG deposition in renal allografts from unrelated rabbits by immunofluorescent technique and tried to correlate our findings with light microscopic alterations in the allografts The study also provide a baseline necessary for evaluation of IgG deposition in renal allografts from recipients sensitized against the kidney donor before transplantation (*Lund & Sommer Hansen VII*, 1972 *Lund, Sommer Hansen & Ahrons* 1972)

Received 9 x 71

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MATERIAL AND METHODS

Random bred, adult New Zealand white rabbits brown lop-eared rabbits and Black Alaska rabbits were used The rabbits weighed between 2 and 3 kg and both female and male animals were used The donor and recipient were chosen from different outbred strains

Kidney transplantation was performed using end to-side anastomosis between the renal vessels of the donor kidney and the abdominal aorta and vena cava inferior of the recipient The ureter was anastomosed to the bladder (*Lund* 1970 *Lund & Myhre Jensen* III 1970) Immunosuppressive therapy was not used Tissue specimens from 74 kidney allografts removed 1 14 days after trans plantation were examined by light and fluorescent microscopy Wedge biopsies alternately from the upper and the lower part of the kidney were obtained from 4 of the allografts 1 hour to 4 days after transplantation The following kidneys served as controls a) 16 autografts removed 1–14 days after transplantation b) 34 of the recipients own kidneys removed together with the allografts and c) 3 normal non transplanted kidneys

Tissue for fluorescent microscopy was frozen im mediately in isopentane cooled to —70° C and stored on blocks of dry ice until further prepara

TABLE 1. *IgG Deposits in Relation to Light-Microscopic Alterations*

day	rabbit no	lymphocyte fluores	lymphocytes	glomerular fluores	proliferative glomerulitis	exudative glomerulitis
1/24	443′	—	+	—	—	—
	444′	—	+	—	—	—
	446′	—	—	—	—	—
1	420	—	—	—	—	—
	434	—	—	—	—	—
	435	—	+	—	—	—
	436	—	—	—	+ + +	+
	443′	+	+	—	—	—
	444′	+	+	—	+ +	—
	445′	+	—	—	—	—
2	69	+	+	—	—	—
	405	+	+	—	—	—
	408	+	+ +	—	+ +	—
	425	—	+	—		—
	428	+	+	—	+ + +	—
	439	+	+	—	—	—
	143	+	—		+ +	—
	444′	—	+	—	+ +	—
	445	—	—	-		—
3	98	—	+	—	+ + +	—
	155	—	+	—	-	—
	407	+	+	—	+ +	—
	412	+	+ +	—	+ +	—
	427	+	+ +	—	+	—
	430	+	+	—	—	—
	444	+	+ +	—	+	—
	446	—	—	—	+ + +	—
4	78	+	+ +	—	+	—
	99	—	—	—		—
	190	—	+ +	—	+	—
	415	+	+ +	—	+ +	—
	416	+	+		—	—
	432	+	+ +	+	+	—
	433	+	+ +	—	+	—
	440	+	—	—	—	+ + +
5	105	+	+ +		+ + +	+ + +
	128	—	+ + +	—	+ + +	+ +
	403	+	+ +	+	+ +	+
	404	+	+ +	—	+ +	—
	406	+	+ +		- + +	+
	442	+	+ +	—	+ +	—
6	112	+	+ + +	+	+ + +	—
	121	+	—	—	—	—
	150	+	+ +	—	+ + +	+ +
	151	—	+	—	—	—
	153	+	+ +	—	+ + +	—
	196	+	+ +	—	+ +	+ +
	197	+	+ + +	—	+ + +	+
	437	+	+ +		+	—

81 Specimens from 74 Renal Allografts Biopsies are Marked by

media fluores	endarteritis	vasculitis	necrosis	micro-thrombosis	tubular fluores
—	—	—	—	—	+
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	+
—	—	—	+++	—	+
—	—	—	—	—	+
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	++	—	—
—	—	—	—	—	—
—	+	—	—	—	+
—	—	—	—	—	+
—	—	—	—	—	+
+	—	—	—	—	+
—	—	—	—	—	+
—	—	—	—	—	+
—	—	—	—	—	—
+	—	—	—	—	—
+	—	—	—	—	+
—	—	—	—	—	—
—	++	—	—	—	+
—	+	+	—	—	—
—	—	—	—	—	+
—	—	—	—	—	—
+	—	—	+	—	—
+	—	—	—	—	—
—	—	—	—	—	—
—	+	—	—	—	—
—	++	++	+	—	+
—	—	—	—	—	—
+	—	—	—	—	+
—	—	—	—	—	+
—	—	+++	+++	+++	—
+	+++	++	—	—	+
—	+++	+++	—	+	+
—	—	—	—	—	+
	+	—	—	—	—
—	+++	++	—	—	—
—	+	—	—	—	+
—	+++	++	—	—	—
—	—	—	+++	+++	—
	+++	+++	—	—	—
	—	—	+++	—	—
—	+	—	—	—	—
+	+	+	++	++	—
	+	+	—	—	—
+	+	++	—	—	—

TABLE 1

day	rabbit no	lymphocyte fluores	lymphocytes	glomerular fluores	proliferative glomerulitis	exudative glomerulitis
7	126	+	+ +	—	+ +	—
	154	+	+ +	—	+ +	—
	163	+	+	—	+	—
8	120	—	—	+	—	—
	120	+	+ + +	—	+ +	—
	131	+	+ + +	—	+ + +	+
	137	+	+ + +	—	+ + +	—
	139	+	+ + +	—	+ + +	+
	141	+	+ +	—	+ + +	—
9	107	+	+ +	—	+ +	—
	143	+	+ +	—	+ +	—
	144	+	+ + +	+	+ +	+ +
	149	+	+ + +	—	+ +	+ +
10	148	+	+ + +	—	+ + +	—
	156	+	+ + +	—	+	—
	159		+ +	—	+ + +	—
	164	+	+ + +	—	+ + +	+ +
	165	+	+ +	—	+ +	—
11	65	+	+ + +	—	+ + +	+
12	185	+	+ + +	—	+ +	+ +
	186	+	+ + +	—	+ + +	+ +
	187	—	+ +	—	+ +	+ + +
	192	+	+ +	—	+	—
	193	—	+	—	—	—
13	422	+	+ +	—	+ +	—
	423	+	+ + +	—	+ + +	+ +
	424	—	+	—		+
	441	+	+ + +	—	+	+ + +
14	413		+ +		+ +	+ +
	414	+	+ +	+	+ +	—
	417		+ +		+	—
	418		+ + +	—	+ + +	+ +

Fluorescence is rated as either + or — The light microscopic alterations are semiquantitatively evaluated

	Mononuclear cell infiltr	Glomerular changes	Vascular changes	Necrosis
+	small infiltrates	in less than 10 % of the glom	in a few vessels	patchy cortial necrosis
+ +	many or large perivascular	in 10 50 % of the glom	in many vessels	subcapsular zone
+ + +	diffuse infiltr	in more than 50 % of the glom	in almost all vessels	partial necr of cortex/medul
+ + + +				total necros s

nued

media fluores	endarteritis	vasculitis	necrosis	micro thrombosis	tubular fluores
—	+	—	—	—	+
—	+	—	—	—	—
—	+	—	—	—	—
+	—	—	+ + + +	+ +	—
—	+	—	—	—	—
—	+ + +	+ +	—	—	—
—	+ + +	+ +	—	—	—
—	+ + +	+ + +	+	+	—
+	+ +	+	—	—	—
+	+	—	—	—	—
—	+	—	—	—	—
—	+ + +	+ + +	+	+	—
—	+	+	+	+	—
—	+	+ +	—	—	—
—	+ + +	+ +	—	—	—
—	+	—	—	—	—
—	+ + +	+ + +	—	+	—
—	—	—	—	—	—
+	+ + +	+ +	—	+ +	—
—	+ +	+ + +	—	—	—
—	+ + +	+ + +	+	—	—
—	+ + +	+ + +	+ + +	—	—
—	+	—	—	—	—
—	—	—	+ + +	—	—
	+ +	+ +	—	—	—
—	+ + +	+ + +	—	—	—
—	+ +	+ +	+	+	—
—	+ + +	+ + +	—	+	—
	—	+ +	+ + +	+	—
—	+ +	+ +	—	—	—
—	—	—	—	—	—
—	+ + +	+ + +	—	—	—

tion Cryostat sections of 6 micron thickness of the unfixed material were washed for 10 minutes in Coon s buffer (pH 7) and incubated *for 30 minutes* in a mo st chamber with goat anti rabbit gamma globulin conjugated to FITC (*Behringwerke RET* 04) Excess of antiglobulin was removed by three wash ngs in Coon s buffer, each lasting for 10 minutes Finally the sections were covered *with* clean coverslips using fluormount (R) as ad hesive The FITC conjugated antiserum was examined by immuno electrophoresis against a panel of 15 sera from rabbits and also against human sera The antisera constantly showed a single arch at the site of IgG against rabbit sera No reaction against human sera was observed The antiserum reacted with the pure IgG fraction of rabbit serum by Ouchterlony s technique

The fluorescein labelled antiserum was used in dilution 1 1 The preparations were examined by dark field fluorescent microscopy using a Zeiss photomicroscope equipped with an ultraviolet high pressure mercury lamp (HBO 200 w) and a dark field condensor Zeiss (NA 1 4) As exiter filters were used BG 38/2 and BG 12/4 and as barrier filters no 44 or 47 Later on a dark field wide angle immersion condensor (Tiyoda) NA 1 4–1 2 was used together with an interference filter (*Rygård & Olsen* 1969) and as barrier filter no 44 or 50

Photographic recording of the preparations was made on high speed daylight ektachrome colour film using Zeiss automatic exposure control *for* ord nary preparations and the same time for block ing test (by manual exposure control) to avoid a

TABLE

day	rabbit no	lymphocyte [illegible]	lymphocytes	glomerular [illegible]	proliferative glomerulitis	exudative glomerulitis
7	[illegible]	+	+ +		+ +	—
	154	+	+ +		+ +	—
	163	+	+		+	—
8	120			+		—
	129	+	+ + +		+ +	—
	131	+	+ + +		+ + +	+
	137	+	+ + +		+ + +	
	139	+	+ + +		+ + +	+
	141	+	+ +		+ + +	
9	107	+	+ +		+ +	
	143	+	+ +		+ +	
	144	+	+ + +	+	+ +	+ +
	149	+	+ + +		+ +	+ +
10	145	+	+ + +		+ + +	
	156	+	+ + +		+	
	157		+ +		+ + +	
	164	+	+ + +		+ + +	+ +
	165	+	+ +		+ +	—
11	65	+	+ + +		+ + +	+
12	185	+	+ + +		+ +	+ +
	186	+	+ + +		+ + +	+ +
	187		+ +		+ +	+ + +
	192	+	+ +			—
	193		+			—
15	422	+	+ +			—
	423	+	+ + +		+	+ +
	424		+			+
	441	+	+ + +			+ + +
14	413		+ +		+	+ +
	414	+	+ +	+	+	—
	417		+ +		+	—
	418		+ + +		+ +	+ +

[illegible] Fluorescence is rated as either + or — The light microscopic alterations are semiquantitatively evaluated

	Mononuclear cell infiltr	Glomerular changes	Vascular changes	Necrosis
+	small infiltrates	in less than 10 % of the glom	in a few vessels	patchy cortical necrosis
+ +	many or large perivascular	in 10–50 % of the glom	in many vessels	subcapsular zone
+ + +	diffuse infiltr	in more than 50 % of the glom	in almost all vessels	partial necr. of cortex/medul
+ + + +				total necrosis



a



b

Fig 2 Renal allograft no 186 removed 12 days after transplantation a mononuclear cell with centrally located nucleus b mononuclear cell with excentric nucleus

nuclei (Fig 1, 2a and 2b) The infiltrates were localized interstitially, primarily around the vessels (Fig 3) and only few lymphocytes with fluorescence were seen intraluminally Lymphocytes with bright fluorescence were found in 4 out of the 16 autografts and in 2 of the recipients' own kidneys removed together with the transplant

Glomerular IgG deposits were observed in 6 allografts (Fig 4 and 5) Five of these grafts also showed light microscopic alterations in the glomeruli while the sixth displayed total necrosis It appears from table 1, however, that 55 allografts showed light microscopic alterations in glomeruli without any IgG deposits The earliest deposits in glomeruli was seen 4 days after transplantation Glomerular fluorescence appeared simultaneously with vascular fluorescence in 2 cases No glomerular fluorescence was observed in kidneys from the control groups

Vascular IgG deposits were seen in the media of small arteries and arterioles in 13 allografts, earliest 2 days after transplantation (Fig 4) Light microscopic examination showed endarteritis (6 cases) and/or vasculitis (5 cases) in 6 of the 13 cases Thirty-nine allografts showed endarteritis or vasculitis without any vascular fluorescence However, in some allografts removed later than 6 days after transplantation with beginning necrosis of the vessel walls, the fluorescent pattern was difficult to discern because the kidney tissue showed diffuse fluorescence Intima fluorescence in blood vessels was seen in 4 of the grafts presenting IgG deposits in media In 2 other cases a bright diffuse brim of fluorescence appeared in the adventitia together with media deposits (Fig 6) Vascular IgG deposition was not observed in the controls

Tubular fluorescence was found in 19 of the 74 allografts as a granular pattern localized basally in the tubular cells The tubular fluorescence was seen during the first four to five days after transplantation, later either no fluorescence or in a few cases a more diffuse fluorescence was observed The tubular fluo-



Fig. 3. Renal allograft no. 40 removed three days after transplantation. Perivascular lymphocytic infiltration.

rescence was observed in some of the autografts as well as in some of the recipients own kidneys removed simultaneously with the graft. In 4 cases only a distinct pattern of fluorescence was seen in the intertubular capillaries. Re-preparations made from tissue blocks which were frozen again after the first preparation and stored at −30° or −70° C were not useful since they as a rule displayed diffuse fluorescent staining of the kidney tissue.

DISCUSSION

Immunoglobulin deposition in renal allografts in dogs (*Burrows et al.* 1963, *Horowitz et al.* 1965), in rats (*Feldman & Lee* 1967, *Lindquist et al.* 1968) and in rabbits (*Heron* 1971) have been reported. These reports are difficult to compare since different techniques have been used and no standardization of the FITC-labelled antigamma-globulin exist. Moreover the frequency of immune deposition has generally not been mentioned.

In human renal allografts suffering rejection crises, about half of the cases presented some sort of immunoglobulin deposition (*Porter* 1967, *Porter et al.* 1968, *Hadley et al.* 1967, *Jeannet et al.* 1967, *Williams et al.* 1967, *McKenzie & Whittingham* 1968).

In our study, the localization of IgG grossly corresponds to that observed in other animals and in human allografts. Variations in the immunofluorescent pattern between different animals and between animals and human grafts are common. In rats f. ex. the mononuclear cells infiltrating the allograft did not show fluorescence (*Feldman & Lee* 1967, *Lindquist et al.* 1968) while lymphocytic infiltrates in renal allografts from dogs (*Horowitz et al.* 1965) and human allografts (*Lindquist et al.* 1968) as well as lympho-

cytic infiltrates in allografts and autografts from rabbits did show fluorescence, supporting the theory that immunoglobulins are bound to the surface of the lymphocytes (*Rabbelino et al* 1970)

The pattern of glomerular fluorescence corresponds to that observed in allografts from dogs Electronmicroscopic observations on allografts from dogs suggest that the gammaglobulin predominantly are localized subendothelially in the glomeruli as reported by *Porter* 1966 who examined tissue specimens from the same allografts by immunfluorescence technique and by electronmicroscopic examination of immunoferritin preparations

In our studies of rabbits, glomerular fluorescence could not be demonstrated earlier than 4 days after transplantation, while light microscopic alterations were seen frequently in allografts removed during the first 3 days after transplantation (Table 1) Glomerular fluorescence was only present in a few allografts presenting light microscopic alterations These findings indicate poor, if any, relation between IgG deposits and histological changes in glomeruli

Vascular deposits of gammaglobulin in intrarenal arterioles in dogs 3 days after transplantation have been reported (*Burrows et al* 1964, *Horowitz et al* 1965) In rats fluorescence was not found in the walls of the arterioles, but in the intrarenal veins (*Lindquist et al* 1968) In human renal allografts immune deposits were rarely found in blood vessels of kidneys suffering rejection crisis during the first 10 days after transplantation while allografts rejected later than 10 days after transplantation frequently presented immune deposits (*Porter* 1967) This later appearance in human materials might be due to immunosuppressive treatment of the recipients or selection according to HL-A

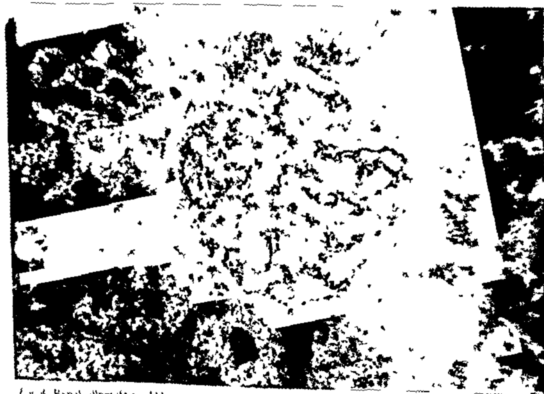


Fig 4 Renal allograft no 444 removed three days after transplantation Slight IgG deposition in glomerulus and in the media of a small arteriole

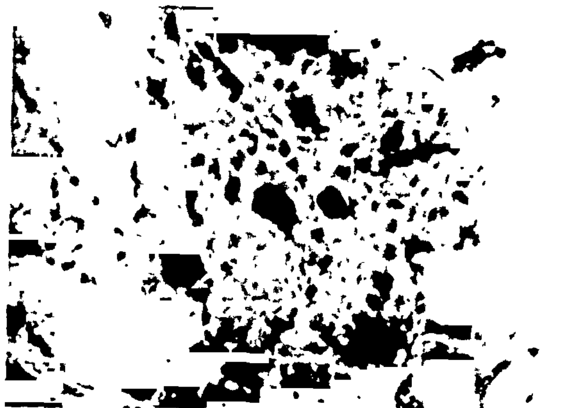


Fig 3. Renal allograft no 112 removed six days after transplantation. Predominantly mesangial localization of IgG in a glomerulus

In our rabbits 6 out of 13 allografts presenting vascular fluorescence showed light microscopic alterations in the blood vessels. However, most of the allografts with endarteritis or vasculitis presented no IgG deposits. In rats immune deposits in the peritubular capillaries of renal allografts have been observed regularly from the second day after transplantation (*Lindquist et al.* 1968). This pattern was only observed in 4 of our rabbits suggesting that peritubular deposition of gammaglobulin is not an important feature in the allograft reaction in rabbits.

The pattern of tubular fluorescence may be related to the strong non specific fluorescence seen in tubular casts, thus representing tubular reabsorption of protein. It is remarkable that tubular fluorescence regularly appeared *during* the first 5 days after transplantation but seldom later. A similar observation has *not* been reported in other animal studies. *There* was no correlation between the intensity of tubular fluorescence and the severity of the allograft reaction.

The allografts from our earliest transplantation were not included because the immunofluorescence study was started later. Some cases were lost because of technical failures. These factors represent a not intended selection, unfortunately excluding some of the more severe histological rejections (*Lund & Myhre Jensen* III 1970). The direct (monolayer) technique used is less sensitive than indirect (two or multilayer) methods (*Nairn* 1969) and the experiments does not rule out that other gammaglobulin fractions (IgA or IgM) may play a *role in* the allograft reaction in rabbits as demonstrated in renal transplantation *in man* (*Porter* 1967) and in rats (*Lindquist et al* 1968). The selection due to technical difficulties, the relative insensitive technique used and *the* limitation of the study to the IgG fraction only, may suggest that the findings *represent*

Fig 6 Renal allograft no 144 removed nine days after transplantation Perivascular fluorescence

a minimum of possible immune deposits in rabbit renal allografts

We must conclude that the technique used failed to demonstrate a correlation between IgG deposition in the renal allografts and the histological appearance of the grafts

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The study was aided by grants from the *Danish Foundation for the Advancement of Medical Science* and *F L Smidth & Co Foundation*

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RENAL TRANSPLANTATION IN RABBITS

VII *IgG Deposits in Allografts from Recipients with Lymphocytotoxic Antibodies Induced by Multiple Skin grafts*

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The frequency, localization and time of IgG deposition have been studied in 48 renal allografts from rabbits with donor specific lymphocytotoxic antibodies prior to kidney transplantation The antibodies were induced by multiple skin grafts The kidney grafts were removed three hours to five days after transplantation Glomerular and/or vascular IgG deposits were found in 25 of the 48 allografts Ten of these 25 allografts presented histological evidence of hyperacute or accelerated rejection five other allografts were removed because of clinical hyperacute rejection but were without histological signs of rejection, and the last ten allografts with IgG deposits had alterations similar to those found in allografts from non sensitized rabbits In the 23 allografts without glomerular or vascular IgG deposits hyperacute or accelerated rejection was demonstrated by light microscopy in 12 cases three allografts were removed because of clinical rejection, but were without histological signs of rejection and the last eight allografts had alterations similar to those in allografts from non sensitized rabbits The frequency of glomerular IgG deposits was greater and the deposits appeared earlier in allografts from sensitized than in allografts from non sensitized rabbits The apparantly increased frequency of vascular IgG deposits was not statistically significant No constant relation was found between IgG deposits and lymphocytotoxic antibodies or histological alterations in the allografts—even in cases with accelerated or hyperacute rejection

In 1966 *Kissmeyer Nielsen et al* reported two cases with hyperacute rejection of a renal allograft In both cases, circulating antibodies to donor leucocytes and thrombocytes were demonstrable at the time of transplantation These antibodies were induced by multiple blood transfusions or by pregnancies Later on, about 80 cases of human hyperacute rejection in presensitized recipients have been reported Presensitization of animals has been shown to result in accelerated (*Simonsen et al* 1953 *Klassen & Milgrom* 1971 *Lund & Myhre Jensen* IV 1971) or hyperacute rejection of kidney allografts (*MacDonald et al* 1970, *Loewenhaupt & Nathan* 1970, *Lund & Myhre Jensen IV*, 1971) Sensitization of rabbits by multiple skin grafts induces lymphocytotoxic antibodies in serum (*Lund & Ahrons* 1972) Some of the lymphocytotoxic antibodies probably belong to the IgG fraction of gammaglobulins (*Colobani et al* 1964, *Walford et al* 1965, *Engelfried* 1966) The intention of the present work was to study a posible relationship between cytotoxic antibodies, histological lesions and IgG deposits in allografts from presensitized rabbits A previous study of 74 allografts from non sensitized rabbits showed IgG deposits in only a

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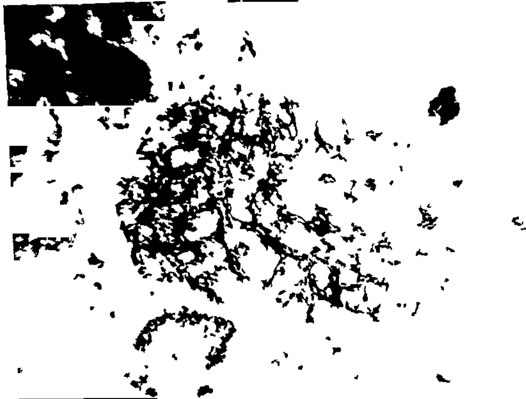


Fig 2 Renal allograft no 230s removed three hours after transplantation IgG deposits in a linear and mesangial pattern Remark the double contours of glomerular capillaries

phocytotoxic antibodies prior to transplantation are shown in table 1

Light microscopic analysis of the allograft reaction in rabbits presensitized by skin grafts has been reported in detail in another paper (*Lund & Myhre Jensen IV* 1971) Briefly the most important alterations were Proliferative glomerulitis (18 cases), exudative glomerulitis characterized by the exudation of neutrophils into glomeruli (16 cases) endarteritis (8 cases), vasculitis characterized by exudation of neutrophils into the vascular walls (5 cases) multiple microthrombosis in glomeruli and small intrarenal arterioles and cortical necrosis (12 cases) Seven of these 12 allografts also presented neutrophilic exudation into glomeruli Perivascular infiltrations with lymphocytes was seen in 17 cases Thirteen renal allografts showed no histological alterations except oedema of varying degree Eight allografts presented some necrosis of the kidney—as a rule in combination with thrombosis of the intrarenal blood vessels (but without multiple microthromboses)

Immunofluorescent Examination

Glomerular IgG deposits were observed in 16 of the 48 allografts Thirteen of these 16 allografts were removed during the first two days after transplantation In four cases the deposits were recognized two to three hours after transplantation The deposits appeared as a linear fluorescence following the contours of the glomerular capillaries or as a granular fluorescence in mesangial regions (Fig 1 and 2)

Glomerular IgG deposits were seen in seven out of 18 allografts with proliferative glomerulitis in six out of 16 cases presenting

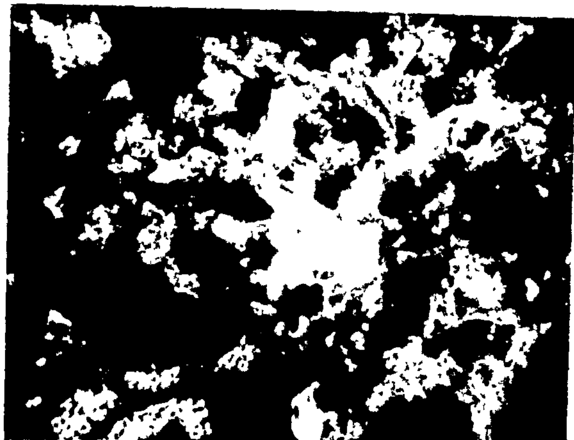


Fig 3 Renal allograft no 241 removed two days after transplantation. Granular ... fluorescent IgG deposits in glomerulus and afferent arteriole

exudative glomerulitis, in six out of 12 allografts with multiple glomerular microthrombi, in two grafts with partial cortical necrosis (but without other signs of rejection) and in five allografts without glomerular or vascular lesions. In some of the allografts with glomerular IgG deposits a combination of proliferative and exudative glomerulitis and microthrombosis was found (Fig 3). Four of the 12 second-set allografts presented glomerular IgG deposits. Two of the recipients' own kidneys removed simultaneously with the kidney graft also had glomerular IgG deposits.

Vascular IgG deposition was found in the media of small arteries and in arterioles in 16 allografts (Fig 4) at the earliest three hours after transplantation. In two cases presenting media fluorescence fine deposits of IgG were also seen on the intima (Fig 5 and 6). In three other cases a dense brim of fluorescence was observed in the adventitia. Vascular deposits were recognized in five out of eight cases presenting endarteritis, in three out of five cases with vasculitis and in eleven allografts without vascular lesions. The combination of endarteritis and vasculitis is shown in Table 1. Four out of the 12 second-set allografts had vascular IgG deposits. Vascular IgG deposition was not found in any of the recipients' own kidneys. In seven of the 16 allografts with vascular IgG deposits glomerular deposits were also found.

A bright fluorescence was generally observed in infiltrating mononuclear cells (17 allografts). The number of infiltrating mononuclear cells with fluorescence was generally less than the number of infiltrating cells observed by light microscopy. Fluorescence was demonstrated in small lymphocytes as well as in larger mononuclear cells with abundant



Fig 4 Renal allograft no 232s removed one day after transplantation IgG deposits in media

cytoplasma and excentric nuclei The cellular infiltrates were generally localized to peri vascular areas near the corticomedullary zone (Fig 5 and 6)

Tubular fluorescence was observed in 18 allografts and appeared a few hours after transplantation In most of these allografts, casts with non specific fluorescence were also found No IgG deposits were observed in peri tubular capillaries

DISCUSSION

We have previously studied IgG deposition in 74 renal allografts from non sensitized rabbits (*Lund & Sommer Hansen VI*, 1972) Among grafts that were removed from one to 14 days after transplantation, only six had glo merular deposits and 13 vascular deposits of IgG No correlation between the light micro-scopic alterations and the glomerular and vas cular deposits was seen

In the present experiments, rabbits were presensitized with multiple skin grafts in order to induce hyperacute rejection of the kidney allografts Sensitization by skingrafting is known to give rise to lymphocytotoxic anti-bodies in serum of the recipients In our experiment, the development of these anti bodies was studied throughout six months prior to kidney transplantation (*Lund & Ahrons* 1972) and antibodies were found in most of the rabbits at the time of transplanta tion As lymphocytotoxic antibodies have been found in the IgG fraction of immunoglobulins (*Colobani et al* 1964, *Walford et al* 1965, *Metzger & Stegler* 1967), one could expect to find these antibodies in kidney grafts using a fluorescent antibody technique with FITC labelled anti rabbit IgG

TABLE 3. [illegible] (*continued*)

Day	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	+		+		
	215			+	+ +	
	[illegible]			+		
	235			+		
[illegible]	[illegible]					
	[illegible]	+				−
	[illegible]					
	245					
	[illegible]	+		+		−
	214	+				
	[illegible]					
	224					
	[illegible]	+			+ +	+
	[illegible]			+	+ + +	+ +
[illegible]	[illegible]					
	[illegible]	+	+		+	
	[illegible]					
	[illegible]					
	[illegible]			+		+ +
	[illegible]				+ + +	+ +
	[illegible]			+		−
	212	+		+		−
	221					−
	222					+ + +
	230		+ +		+ +	−
	[illegible]			+	+ +	+ +
2	[illegible]	+	+	+		−
	247	+	+ +		+ +	+ + +
	248	+	+ +	+	+ +	+ +

ensiti ed by Multiple Skin Grafts Fluorescence is Marked + Key for Lightmicroscopic Evaluation is Table

media fluores	endarteritis	vasculitis	necrosis	micro thrombosis	tubular fluores	cytotoxic antibody titre
—	—	—	—	—	—	32
—	—	—	—	—	+	16
—	—	—	—	—	+	—
—	—	—	—	—	+	8
—	—	—	—	—	—	—
+	—	—	—	—	—	—
—	—	—	—	—	—	—
—	—	—	—	—	+	8
—	—	—	—	—	+	—
—	—	—	—	—	—	16
—	—	—	—	—	—	—
—	—	—	+ + +	+ +	—	1
+	+ +	—	+ +	+	—	2
—	—	—	+ + +	+ +	+	—
+	—	—	—	—	—	—
—	+	—	—	—	+	—
—	—	—	+ + +	—	—	—
—	—	—	—	—	—	—
+	—	—	—	+ +	+	—
—	—	—	—	—	+	—
—	—	—	+ + +	—	—	—
—	—	—	—	—	—	1
—	—	—	+ + +	+	—	—
—	—	—	+ + +	+ + +	—	—
+	—	—	—	—	+	1
+	—	—	+ + +	+ +	+	4
+	—	—	—	—	+	1
+	+ +	+	+ +	+ + +	—	32
+	—	—	—	—	+	8
—	—	—	—	—	—	1
+	—	—	+ + +	—	—	1
—	—	—	—	—	—	2
—	+ +	+ +	—	—	—	1
—	—	—	+ + + +	+ + +	—	—
—	—	—	+ + +	+ + +	—	8
+	+	+	—	—	—	1
—	+	+	+	+	+	8
+	—	—	—	—	+	1
+	—	—	+ + +	+ +	+	4
—	—	—	—	+	+	2
+	+ +	—	+ + +	+ + +	—	4
+	+ +	+	—	—	—	—
	—	—	—	—	+	—
	—	—	+ + +	+ +	—	8
—	—	—	+ + + +	—	—	4
—	—	—	+ + +	+ +	—	2
+	—	—	+ + +	—	—	2
—	—	—	+ + +	—	—	4

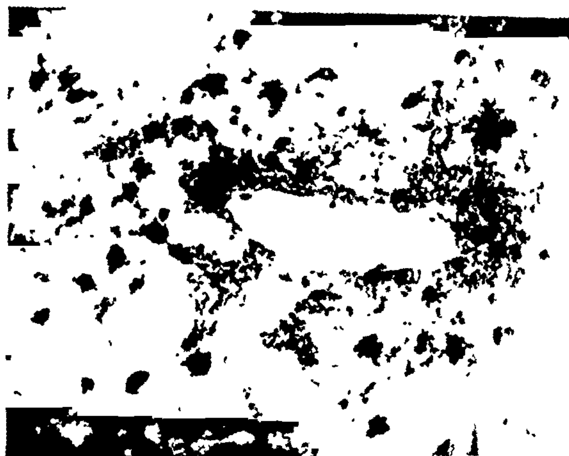


Fig. 5 Renal allograft no 250 removed three days after transplantation. Intrarenal bl od vessel present ng vasculitis. IgG deposits in intima, media and in perivascular cell infiltrates

Key for semiquantitative evaluation

	mononuclear cell infiltr	glomerular changes in	vascular changes in	necrosis
	small infiltrates	less than 10 % of the glom	a few vessels	patchy cortical necrosis
+ +	many or large perivascular	10–50 % of the glomeruli	many vessels	subcapsular zone
+ + +	diffuse infiltr	more than 50 % of the glomeruli	in almost all vessels	partial necrosis of cortex and medulla
+ + + +				total necrosis

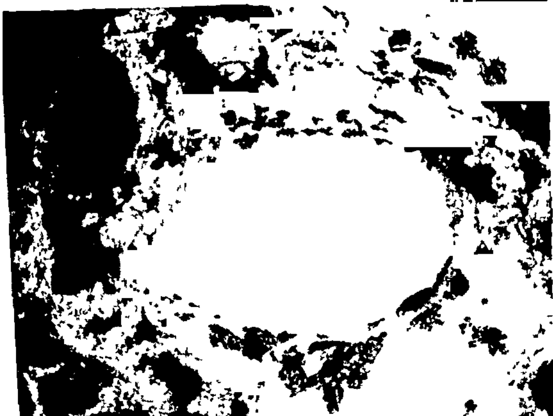


Fig 6 Same allograft as in fig 5 Intima and media fluorescence

TABLE 2 *The Frequency of IgG Deposition in Renal Allografts from Non-Sensiti ed and Pre-Sensiti ed Rabbits The Allografts Were Removed One to Three Days after Transplantation*

	Total number	Glomerular fluores	Vascular fluores	Lymphocytic fluores	Tubular fluores
Non sensitized	20	0	4	14	12
Pre-sensitized	35	11	14	17	14
		Fischer ($P<0\ 05$)	χ^2 ($P>0\ 05$)	χ^2 ($P>0\ 05$)	χ ($P>0\ 05$)

Compared with allografts from non sensitized rabbits glomerular and vascular deposits appeared *earlier and glomerular deposits* more frequently in the sensitized group The deposits could already be seen a few hours after transplantation (in the nonsensitized group after two to three days), in fact 10 out of the 25 allografts with glomerular or vascular deposits presented IgG deposition with*in the first two days after transplantation* In Table 2 a comparison is given between allografts from sensitized and non sensitized allografts removed during the first three days after transplantation Allografts in represen-

itative numbers were not available after the third day. It appears from the table that the frequency of fluorescence in perivascular mononuclear cells and in tubular cells was almost similar in the two groups. Glomerular fluorescence was significantly more frequent in the sensitized group whereas the apparantly increased frequency of vascular IgG deposits was not significant (Table 2).

Histological examination of the kidney allografts from sensitized and non sensitized rabbits showed that two of the alterations appeared earlier in the sensitized group, namely exudation of neutrophils into glomeruli or the walls of intrarenal blood vessels and multiple glomerular microthrombosis (*Lund & Myhre Jensen III & IV*, 1970/71). In the following discussion, appearance of neutrophils in glomeruli or intrarenal blood vessels during the first three days is considered an indicator of accelerated rejection and presence of glomerular microthrombosis an indicator of hyperacute rejection.

Twenty five out of the 48 allografts had glomerular or vascular deposits of IgG (Table 1). Ten of these 25 allografts had histological signs of hyperacute or accelerated rejection. Four allografts showed *clinical* hyperacute rejection within the first three hours after transplantation but no histological signs of rejection. The last ten allografts with IgG deposits had histological alterations similar to those seen in allografts from nonsensitized rabbits.

Twenty three out of the 48 allografts were without glomerular or vascular deposits. Twelve of these allografts presented histological signs of hyperacute or accelerated rejection, four allografts were removed because of clinical rejection but showed no histological signs of rejection and in the last eight grafts alterations were found to be similar to those in the non sensitized group removed at the same time after transplantation. As IgG deposits were observed in about half of the allografts and appeared earlier and as histological alterations also were seen earlier and were more widespread in the presensitized group, a certain relationship between IgG deposits and the lesions observed could be expected to be similar to features observed in animal models where passive transfer of donorspecific hyperimmune serum results in vascular damage and rapid destruction of the graft (*Najarian & Perper* 1967, *Dubernard et al.* 1968). *Najarian & Perper* demonstrated IgG deposition in the blood vesels in these kidneys. Less than half of our allografts with glomerular or vascular lesions had, however, IgG deposits at the time of examination. A possible explanation of this low frequency could be that IgG deposits in presensitized rabbits had appeared early after transplantation and had disappeared again at the time of examination. The greater frequency of IgG deposits in grafts removed during the first two days after transplantation than in grafts removed later supports this possibility. In human kidney allografts *Busch et al.* 1971 found a correlation between rapid rejection of the graft, infiltration with polymorphonuclear leucocytes and IgG deposits in intrarenal arteries but no correlation between *glomerular* IgG deposits and the allograft reaction. About 80 cases of human hyperacute rejection have been reported but only a few of these have been studied by immunofluorescent technique. Glomerular deposits have been observed by *McKenzie & Whittingham* 1968, *Busch et al.* 1969 and 1971, vascular deposits by *Busch et al.* 1971 and pertubular fluorescence by *Williams et al.* 1967.

Others for instance *Starzl et al.* 1968 and *Myburgh et al.* 1969 did not find IgG deposition in cases of hyperacute rejection.

Animal studies of hyperacute or accelerated rejection of kidney allograft in presensitized animals do not mention immunofluorescent studies (*Loewenhaupt & Nathan* 1968 (dogs), *Klassen & Milgrom* 1969 (rabbit) and *Mac Donald et al.* 1970 (rabbits)). In our experiments six out of 12 rabbits with hyperacute rejection had IgG deposits in glomeruli or in intrarenal blood vessels. It is remarkable that five of these six cases had exudation of neutrophils which was not found in any of the cases without IgG deposits. This might suggest a relationship between IgG deposition and neutrophils in the most

severe reactions Unfortunately nine of the original 57 allografts from sensitized rabbits were not available for immunofluorescent study because of technical reasons, thus making a non-intended selection on the material (five out of these nine grafts showed hyperacute rejection pattern on light microscopic examination)

Deposition of IgG in grafts with minimal histological alterations could represent a stage with antigen antibody interaction just prior to a stage with histological lesions, but could also be due to enhancing antibodies stronger than the lymphocytotoxic antibodies demonstrated in serum of recipients at the time of transplantation

Observations in rabbits presensitized by multiple injections of donorspecific kidney homogenate have shown that even though all recipients (15) developed lymphocytotoxic antibodies only two had histological signs of hyperacute rejection and none had accelerated rejection Ten of these 15 allografts had IgG deposits in glomeruli or intrarenal blood vessels with only minimal histological changes even in daily biopsies taken during the first six days after transplantation In these rabbits enhancing antibodies had probably been induced by the kidney homogenate (*Lund et al*)

Skin grafting is known to induce antibodies other than cytotoxic ones (*Amos et al* 1954 *Gorer & O Gorman* 1956 *Stetson & Jensen* 1960 *Kapitchnikov et al* 1962) It is possible that formation of enhancing or blocking antibodies explain these cases with demonstrable IgG deposits but without histological lesions in the group of rabbits presentized by skingrafting Our experiments do not exclude the possibility that other fractions of gamma globulins (IgM or IgA) may play a role in the rejection of allografts from sensitized recipients Actually the IgM fraction has been shown to have lymphocytotoxic activity too (*Stocker et al* 1969 *Ahrons & Glassen Kristensen* 1971)

In conclusion the experiments with kidney grafts from skinsensitized rabbits showed an earlier and more frequent deposition of IgG in glomeruli and an earlier deposition of IgG in vessels than in grafts from non-sensitized rabbits No constant correlation between IgG deposits and light microscopic observations was found, however, as only half of the grafts with histological lesions had IgG deposits

The study was aided by grants from the *Danish Foundation for the Advancement of Medical Science* and *F L Smidth & Co Foundation*

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Acta path. microbiol. scand. Section A. 80: 403-411, 1972

RENAL MICROTHROMBOSIS

Incidence in 500 Consecutive Autopsies. Clinico-pathological Relations

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In a study of 500 consecutive autopsies, 400 in a hospital department for morbid anatomy, 100 in an institute of forensic medicine, microthrombosis was found in 12 per cent of the kidneys (13 per cent of the hospital series, 7 per cent of the forensic series). In none of the positive cases were renal microthrombosis suspected from clinical evidence and macroscopial appearance. None of the kidneys had focal or symmetrical cortical necrosis. A positive association between renal microthrombosis and thrombo-embolic disease, severe infections, malignancies, diabetes mellitus, various conditions with evidence of a haemorrhagic diathesis, treatment with an antifibrinolytic agent (epsilonaminocaproic acid) and steroid hormones and ACTH indicates its biological significance. A correlation which is statistically significant at a high level between renal microthrombosis in its more severe degrees and thrombo-embolic disease, most cases being venous thrombosis, probably reflects a state of hypercoagulability in large venous thrombosis. The lack of late manifestations of renal microthrombosis, i.e. focal or symmetrical cortial necrosis, indicates that intravascular coagulation was a rather terminal event most probably related to the general breakdown of homeostasis in the agonal period. The demonstration of microthrombosis, a morphological sign of intravascular coagulation, is of importance for evaluation of cases with severe disturbance of the haemostatic equilibrium. If not searched for, however, microthrombosis may easily be overlooked.

Intravascular coagulation seems to be an important intermediary pathogenic mechanism in several diseases. Fulminant syndromes of disseminated intravascular coagulation are clinically characterized by a consumption coagulopathy and morphologically by widespread platelet- and fibrinthrombi in small blood vessels (*McKay* 1965). The generalized Shwartzman reaction in the rabbit produces a bilateral renal cortical necrosis, supposed to be secondary to a massive renal cortical microthrombosis. Shwartzman like syndromes in man are fairly rare, about one case being encountered in 1000 autopsies (*Skjörten* 1966).

Received 4.xi.71

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Renal microthrombosis without overt cortical necrosis, however, might be found more frequently in an autopsy material.

In order to estimate the incidence at autopsy and clinicopathological relations of renal microthrombosis, 500 consecutive autopsies were studied. Having collected the material, two publications on this subject appeared (*Harms & Lehmann* 1969, *Skjörten* 1969). Our results support and further extend their observations.

MATERIAL AND METHODS

Specimens were sampled by one of the authors (BB) from 500 consecutive autopsies performed

TABLE 1 *Distribution in Percentage According to Age and Sex in 500 Consecutive Autopsies*

Age (years)		0 9	10 19	20 29	30 39	40-49	50-59	60 69	70-79	≥ 80	Total
Hospital series	F	5	1	1	2	2	7	8	13	8	47
	M	3	1	1	2	4	6	14	14	8	53
Forensic series	F	8	2	3	2	6	2	5	4	0	32
	M	8	9	7	9	6	15	10	4	0	68

TABLE 2 *Distribution in Percentage According to the Interval between Death and Autopsy*

Interval (hours)	Hospital series	Forensic series
0 11	0	6
12-23	32	21
24-47	50	55
48-71	15	9
72 95	2	3
96	1	6

during a half year period As we wanted to examine both a series of cases from a large hospital autopsy and a series including autopsies of healthy persons killed in accidents we studied a consequtive series of 400 autopsies from the department of pathology and 100 from the department of forensic medicine The composition of the material according to age and sex to interval between death and autopsy and to mode and cause of death is demonstrated in Tables 1-3

In each autopsy only one specimen was taken always as far as possible from an uscarred area in one of the kidneys The tissue was fixed in 4 per cent neutral, phosphate buffered formaldehyde solution paraffin embedded and cut at about 6 8 µ Sections were ... (HE), van ... haematoxylin ... toxylin (PTAH) Lendrum s and Ladewig s techniques for fibrin

The stained sections, usually about 3 4 cm² were coded and then screened blindly for the presence of thrombi in the intrarenal blood vessels (arcuate, interlobular vasa rectae, glomerular and peritubular capillaries) by two of the authors (OMJ, BB) Based on the PTAH stain findings were semiquantitated O no thrombi, 1+ an occasional thrombus per section 2+ thrombi in up to ten vessels and glomeruli per section 3+, thrombi in more than ten vessels and glomeruli per section, but not in all, 4+, disseminated microthrombosis involving almost every vessel and glomerulus of the section A few sections were registered as doubtful positive (?)

Case histories and autopsy reports were studied in retrospect, and the data to be related to the findings of renal microthrombosis were registered by one of the authors (ESH) who was not involved in the screening for renal microthrombosis at the time of registration The following states were noted disturbance of blood coagulation disturb- ... treatment, anti ... erapy ... bo-embolism except coronary thrombosis, coronary occlusion including thrombosis of the coronary arteries, malignant neoplasm leukæmia and carcinoma of the pancreas and prostata considered apart acute infectious disease septicaemia considered apart pregnancy steroid therapy (sexual hormones, cortisone) and ACTH blood transfusion haemolysis extracorporeal circulation acute haemorrhagic necrosis of pancreas hyperparathyroidism severe liver damage renal disease (glomerulonephritis pyelonephritis infarction) including disseminated lupus erythematosus malignant hypertension and diabetes mellitus A few cases in which these states were not definitely absent or present were registered as unknown

The correlation between clinico-pathological data and renal microthrombosis was tested by the Chi square method in Yates modification (Table 5)

RESULTS

The incidence of renal microthrombosis is 12 per cent in this material In the hospital autopsies the incidence is definitely higher, 13 per cent than in the forensic autopsies, 7 per cent The corresponding figures for the most severe degrees of microthrombosis (4 and 3+) are 5 per cent and 1 per cent (Table 4) In positive specimens thrombi

TABLE 3 *Distribution of 500 Consecutive Autopsies According to Mode and Cause of Death*

Mode of death	Cause of death	Hospital series no	Hospital series %	Forensic series no	Forensic series %
Natural death	cardiovascular	150	38	18	18
	malignancies	105	26	0	0
	CNS	43	11	6	6
	respiratory system	30	8	4	4
	digestive system	29	7	1	1
	urogenitary system	22	6	0	0
	miscellaneous	10	2	4	4
	total	389	98	33	33
Accidental death	lesions of head neck	9	2	30	30
	lesions of trunk pelvis	1	0	8	8
	multiple lesions	1	0	8	8
	miscellaneous	0	0	9	9
	total	11	2	55	55
Suicide	poisoning			2	2
	drowning			2	2
	miscellaneous			2	2
	total	0	0	6	6
Homicide	strangulation, etc			3	3
	drowning			2	2
	miscellaneous			1	1
	total	0	0	6	6

were most often located in glomerular and interlobular vessels The vasa rectae of the renal medulla contained only few thrombi even in highly positive cases Not all thrombi gave a positive reaction in the Lendrum and Ladewig stains In the PTAH stain some erythrocytes stained a clear blue, but thrombi were usually more intensely darkblue or violet and granular or fibrillar in texture Renal microthrombosis was not suspected from the macroscopical appearance of the kidneys in any case, not even in those with multiple, extensive microthrombi None of the positive cases had renal cortical necrosis indicating that intravascular coagulation was most probably a terminal event Roughly estimated the renal morphological alterations, including hyperaemia, interstitial oedema tubular epithelial degeneration or autolysis were similar in cases with renal microthrombosis and in cases without. The presence of renal microthrombosis was not correlated with age and sex, interval between death and autopsy, and the mode and cause of death The youngest

TABLE 1 *Distribution in Percentage According to Age and Sex in 500 Consecutive Autopsies*

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	M	3	1	1	2	4	6	11	14	8	53
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during a half year period As we wanted to examine both a series of cases from a large hospital autopsy and a series including autopsies of healthy persons killed in accidents, we studied a *consecutive series* of 400 autopsies from the department of pathology and 100 from the department of forensic medicine The composition of the material according to age and sex to interval between death and autopsy, and to mode and cause of death is demonstrated in *Tables 1 3*

In each autopsy only one specimen was taken *always as far as possible from an uscarred area in* one of the kidneys The tissue was fixed in 4 per cent neutral phosphate buffered formaldehyde solution paraffin embedded and cut at about 6–8 μ Sections were stained with haematoxylin-eosin (HE) van Gieson (vG) periodic acid Sluff haematoxylin (PAS), phosphotungstic acid haematoxylin (PTAH) Lendrum s and Ladewig s techniques for fibrin

The stained sections usually about 3–4 cm² were coded and then screened blindly for the presence of thrombi in the intrarenal blood vessels (arcuate, interlobular vasa rectae glomerular and peritubular capillaries) by two of the authors (OMJ, BB) Based on the PTAH stain, findings were semiquantitated O no thrombi 1+ an occasional thrombus *per section* 2+, *thrombi in up to ten* vessels and glomeruli per section 3+, thrombi in more than ten vessels and glomeruli per section, but not in all, 4+, disseminated micro*thrombosis involving almost every vessel* and glomerulus of the section A few sections were registered as doubtful positive (?)

Case histories and autopsy *reports* were studied in retrospect, and the data to be related to the findings of renal microthrombosis were registered by one of the authors (ESH) who was not involved in the screening for renal microthrombosis at the time of registration The following states were noted disturbance of blood coagulation disturbance of fibrinolysis anticoagulant treatment anti fibrinolytic treatment, systemic fibrinolytic therapy evidence of haemorrhagic diathesis, thrombo-embolism *except coronary* thrombosis coronary occlusion including thrombosis of the coronary arteries, malignant neoplasm leukæmia and carcinoma of the pancreas and prostata *considered* apart acute infectious disease septicaemia considered apart, pregnancy, steroid therapy (sexual hormones, cortisone) and ACTH blood transfusion, haemolysis extracorporeal circulation acute haemorrhagic necrosis of pancreas hyperparathyroidism severe liver damage renal disease (glomerulonephritis pyelonephritis infarction) *including* [illegible]

were registered as unknown

The correlation between clinico-pathological data and *renal microthrombosis* was tested by the Chi square method in Yates modification (*Table 5*)

RESULTS

The incidence of renal microthrombosis is 12 per cent in this material *In the* hospital autopsies the *incidence* is *definitely higher,* 13 per cent, than in the forensic autopsies 7 *per cent* The corresponding figures for the most severe degrees of microthrombosis (4 and 3+) are 5 per cent and 1 per cent, (Table 4) In positive specimens, *thrombi*

TABLE 5 *Relationship between Renal Microthrombosis at Autopsy and Various Clinical and Patho Anatomical States*

		Degree of renal microthrombosis				
		++++ +++	++ +	?	0	Total
Coagulation	normal	14	32	5	353	404
	abnormal	4	4	0	53	61
	? normal	2	4	0	29	35
Anticoagulant treatment	not given	18	38	5	399	460
	given	2	2	0	34	38
	? given	0	0	0	2	2
Haemorrhagic diathesis	not present	18	38	5	424	485
	present	2	2	0	9	13
	? present	0	0	0	2	2
Thromboembolism	not present	8	31	5	398	382
	present	12*	9	0	93	114
	? present	0	0	0	4	4
Coronary occl myocard infarct	not present	19	34	4	376	433
	present	1	6	1	58	66
	? present	0	0	0	1	1
Malignancy	not present	13	27	5	313	358
	present	7	13	0	116	136
	? present	0	0	0	6	6
Severe infection	not present	11	21	2	283	317
	present	9	18	3	150	180
	? present	0	1	0	2	3
Steroid therapy	not given	17	32	4	367	420
	given	3	6	0	44	53
	? given	0	2	1	24	27
Blood transfusion	not given	11	32	5	299	347
	given	9	8	0	128	145
	? given	0	0	0	8	8
Liver damage severe	not present	20	37	5	410	472
	present	0	3	0	21	24
	? present	0	0	0	4	4
Diabetes mellitus	not present	18	36	5	403	462
	present	2	4	0	32	37
	? present	0	0	0	1	1

* The correlation between renal microthrombosis (4 and 3+) and major thrombo-embolic disease is statistically highly significant (Chi square 15 $P < 0.001$)

Disturbance of blood coagulation Treatment with anticoagulants or antifibrinolytics Evidence of haemorrhagic diathesis—Mild disturbance of blood coagulation in most cases by a moderate decrease in prothrombin proconvertin levels, was diagnosed in 61 cases, all from the hospital series The incidence of renal microthrombosis (8/61, 13 per cent) was identical with that of the entire material Two patients had active fibrinolysis in the days before death, one of them had renal microthrombosis Thirty eight patients were

given anticoagulants in the period up to death four of them (10 per cent) had renal microthrombosis Three patients were treated with an anti fibrinolytic agent (epsilon aminocaproic acid), two had renal microthrombosis None of the patients of the material were given systemic fibrinolytic therapy (streptokinase, urokinase etc) In thirteen of the autopsies various findings indicated an haemorrhagic diatheses renal specimens contained microthrombi in four (30 per cent)

Thrombo embolism — Thrombo-embolic disease other than coronary thrombosis was found in 113 cases of the material 21 cases of these had renal microthrombosis (19 per cent) The positive correlation is highly significant in cases with severe degrees of renal microthrombosis (Chi square 15 $P < 0.001$) but not in cases with slight renal microthrombosis (2 and 1+) The vast majority in this group were patients with venous thrombosis and/or pulmonary thromboembolism Cases with coronary thrombosis as the only indication of thrombo embolic disease were not included

Coronary occlusion and myocardial infarction —Sixty five patients in the material died with coronary occlusion and myocardial infarction In about half of these cases occlusion was caused at least in part—by coronary thrombosis The incidence of renal microthrombosis (11 per cent) was slightly below that for the entire material the difference however is not statistically significant. Ten patients within this group had been treated with anticoagulants Renal microthrombosis was present in one but in a slight degree (1+)

Malignant neoplastic disease —Malignant neoplasm was found in 136 autopsies As the malignancy was not considered cause of death in all cases this figure is not identical with that in Table 3 The incidence of renal microthrombosis (15 per cent) is not significantly increased Ten cases of this group had leukæmia two of them showed renal microthrombosis both in a severe degree Four patients with carcinoma of the prostata and one with carcinoma of the pancreas had *no renal* microthrombosis Seventeen patients were in radiation therapy within a reasonable short period before death four of them had renal microthrombosis (24 per cent) an incidence which is not significantly increased

Severe acute infections —The incidence of renal microthrombosis in this group is 16 per cent The figure, however is not significantly increased when compared with that of the entire material ($P < 0.1$) Septicaemia was diagnosed in thirteen patients two of these (15 per cent) had renal microthrombosis both in severe degree

Blood transfusion Evidence of haemolysis Extracorporeal circulation —Blood transfusions were given to 145 patients in the days before death 17 had renal microthrombosis (12 per cent) Evidence of a moderate haemolysis shortely before death (5 cases) and extracorporeal circulation during operations in heart lung machine (8 cases) was not followed by renal microthrombosis at all

Treatment with steroid hormones and ACTH —Fifty three patients were in hormone therapy in the period up to death nine with renal microthrombosis 17 per cent) The positive correlation however is not statistically significant Two patients were pregnant none had renal microthrombosis

Miscellaneous —Severe liver damage demonstrated by liver function tests and/or post mortem examination was seen in 24 cases of these 3 had renal microthrombosis (13 per cent), Acute haemorrhagic pancreas necrosis was found in 9 cases only one with renal microthrombosis (11 per cent) One patient dying in hyperparathyroidism had no renal microthrombosis Three patients of the entire material had been prostatectomized during the terminal hospitalization One had a questionable renal microthrombosis (?) two had definite renal microthrombosis (3+) in one a severe coagulation defect had been diagnosed in vivo

Renal disease Systemic collagen disease Diabetes mellitus Renal infarction was found in 6 autopsies in which one had renal microthrombosis Three patients had glomerulo-

nephritis in only one a few glomerular thrombi were demonstrated Two cases of suppurative pyelonephritis had no renal microthrombosis Only one patient suffered from systemic lupus erythematosus having no renal microthrombosis Malignant hypertension was not encountered in this material Thirty seven patients had diabetes mellitus, six of these showed renal microthrombosis (16 per cent) three in a severe extent (3+) In those three patients diabetes mellitus had been diagnosed for only a few years and there was no clinical and morphological evidence of diabetic nephropathy Three diabetics with a proven nephropathy had a fairly mild renal microthrombosis (2+)

DISCUSSION

The incidence of renal microthrombosis was 12 per cent in this study a figure which is identical with that of *Harms* & *Lehmann* (1969) 12 3 per cent The low incidence in an autopsy material from a department of forensic medicine dealing with cases of sudden unexpected death, also is in agreement with previous observations (*Remmele* & *Harms* 1968) In his material of 100 consecutive autopsies from a large general hospital *Skjorten* (1969) found hyaline microthrombi (i e intravascular globular eosinofilic bodies with a diameter from about that of an erythrocyte up to 30–40 μ positive in the fibrin stains) in 15 5 per cent of the kidneys The incidence of mural or occlusive platelet and fibrinthrombi in the small intrarenal blood vessels was 9 3 per cent In none of the kidneys in the series of *Harms* & *Lehmann* and *Skjorten* either was renal microthrombosis suspected from clinical and patho-anatomical evidence and renal cortical necrosis was absent The lack of concomittant morphological alterations of the kidneys with microthrombosis indicates that intravascular coagulation was a terminal phenomenon The correlation between microthrombosis at autopsy and certain clinical conditions demonstrates however that intravascular coagulation is not a purely agonal event without biological significance

The association between shock and intravascular coagulation has been demonstrated clinically and experimentally (*Crowell* & *Read* 1955 *Hardaway* 1966), an association that could be found in an autopsy material too (*Harms* & *Lehmann* 1969) The occurrence of shock was not registrered in ourstudy as we felt it impossible to evaluate in retrospect the extent and duration of hypotension Some of our observations for instance the low incidence of renal microthrombosis in sudden deaths, might be explained by this interrelationship Hypotension is part of the breakdown of homoeostasis during agony The duration of the agonal period, therefore might be correlated with the incidence of microthrombosis at autopsy In the studies of *Harms* & *Lehmann* and *Skjörten* a positive association between microthrombosis at autopsy and severe infections malignancies and thrombo embolic diseases was found but was not statistically significant This association was again demonstrated in the present study and furthermore the correlation to thrombo-embolic disease of major blood vessels was statistically significant as to the more severe degree of renal microthrombosis It probably reflects a state of hypercoagulability that might be a pathogenic factor in the thrombotic process or a result from the thrombosis of the large vessels This interpretation is supported by the observations of *Adelson et al* (1961) The induction of a large venous thrombus resulted in a markedly decrease in the number of platelets which could not be explained by the local consumption in the formation of the thrombus Probably the clot released more thrombin than could be neutralized locally or systemically resulting in a state of hypercoagulability Similar results from injection of thrombin (*Quick* 1958) and thromboplastin (*Adelson et al* 1960) are further evidence that the platelet decrease is due to systemic rather than local platelet utilization

The association between intravascular coagulation and various clinical conditions

including severe infections and malignancies is well documented (cf *McKay* 1965 *Miller et al* 1967) Clinically patients with disseminated malignant tumours and leukaemia often have disturbed blood coagulation (*Soong & Miller* 1970 *Brakman et al* 1970 *Pollack* 1971) In this material one of the two cases with a severe renal microthrombosis had leukaemia (Fig 1) this patient was furthermore treated with an antifibrinolytic agent (epsilon aminolaproic acid) Enhanced fibrinolytic activity however may be secondary to intravascular coagulation and the administration of antifibrinolytics might be dangerous because of the delay in clot resolution Complicating intravascular coagulation in patients treated with epsilon aminolaproic acid has been observed (*Naye* 1962 *Neergaard & Thybo* 1970 *Gralnick & Greipp* 1971) That disseminated intravascular coagulation as demonstrated by the presence of microthrombosis is correlated to major thromboembolic phenomena is a new observation The higher incidence of renal microthrombosis although not statistically significant in patients treated with steroid hormones and ACTH is a new observation too A relationship between thromboembolism and oral contraceptive hormones still is much debated although it is known that hormonal imbalances affect blood coagulation factors (*Penick* 1968) Experimentally treatment with cortisone ACTH and other hormones may act as a preparatory mechanism in the generalized Shwartzman reaction (*Thomas & Good* 1952 *Cooper & McKay* 1960 *McKay & Merriam* 1960 *Buitrago & Myhre Jensen* 1968) None of the patients in this material recieved oral contraceptives according to available information

In various diseases of the kidney fibrin is deposited in glomeruli and blood vessels In the present material only few cases with active glomerular or interstitial disease occurred none had renal microthrombosis In the series of diabetics a positive association with renal microthrombosis was found although not statistically significant The kidneys of three diabetics in whom the disease was of rather short duration and without evidence clinically and morphologically of a diabetic nephropathy had pretty extensive thrombosis af the small intrarenal blood vessels Three more cases of diabetes mellitus with a nephropathy had moderate renal microthrombosis In these cases exudative fibrinoid lesions of glomeruli and blood vessels may have been misinterpreted No attempt was done to relate renal microthrombosis to the degree of arteriolar and arterionephrosclerosis No association however between age and renal microthrombosis was demonstrated

This study has shown that renal microthrombosis in autopsy specimens specially from a hospital series is a rather frequent finding when searched for A positive association with thromboembolic disease severe infections malignancies diabetes mellitus treatment with an antifibrinolytic agent (epsilon aminolaproic acid) and steroid hormones and ACTH indicates its biological significance A correlation between thrombo-embolic disease most cases being venous thrombi and renal microthrombosis which furthermore is statistically significant at a high level probably reflects a state of hypercoagulability in large venous thrombosis The lack of late manifestations of renal microthrombosis i e focal or symmetricel cortical necrosis indicates that intravascular coagulation was a rather terminal event most probably related to the general breakdown of homeostasis in the agonal period

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Acta path microbiol scand Section A 80 412–420, 1972

APPLICATION OF POINT-COUNTING TECHNIQUE TO THE QUANTITATIVE ASSESSMENT OF CORONARY AND AORTIC ATHEROSCLEROSIS

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A modification of the point-counting technique applicable to the quantitative assessment of atherosclerotic lesions in the coronary arteries is described The results obtained by this technique are well reproducible and the accuracy of the method is equal to that of planimetry With the point-counting technique described it is possible to assess the extent of atherosclerotic lesions in the coronary arteries with the same accuracy as the extent of atherosclerotic lesions in the aorta is assessed with the point-counting technique described earlier by *Mitchell & Cranston*

The extent of coronary and aortic atherosclerosis has been quantitated in extensive postmortem studies by visual assessment of the percentage of the intimal surface area affected by atherosclerotic lesions (*Uemura et al* 1964, *Guzman et al* 1968, *Tejada et al* 1968) In some investigations visual assessment has been facilitated by drawing over a flattened arterial specimen, enclosed in a plastic bag a diagram dividing the intimal surface area into smaller parts of known size (*Sternby* 1968, *Vihert* 1970) If the technique is carefully standardized, the reproducibility of the visual assessment of the percentage areas of atherosclerotic lesions is relatively good for groups of specimens, but not necessarily for individual specimens (*Uemura et al* 1964, *Guzman et al* 1968, *Sternby* 1968)

A greater accuracy of measurement and better reproducibility of results can be attained with planimetric techniques (*Cranston et al* 1964, *Lichtlitz et al* 1966)

Since area measurement by planimetric techniques is timeconsuming more simple sampling methods have come into use In line sampling area measurements are replaced by length measurements and in point sampling by counting points falling over the areas to be measured These methods have long been used in geographic measurements (*Yates* 1949) Both the line sampling (*Uotila* 1940, *Uotila & Kannas* 1952) and point sampling (*Chalkley* 1943 *Lagerstedt* 1949) have been widely used for the measurement of relative areas in quantitative histological studies *Dunnill* (1962) employed point counting for macroscopic estimation of the extent of pulmonary lesions *Mitchell & Cranston* (1965) adopted this technique from Dunnill and applied it to quantitative assess

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ment of aortic atherosclerosis The method described by *Mitchell & Cranston* is based on the principle of systematic point sampling, i e when a large number of points arranged in a given pattern is superimposed over irregular outlines on a plane surface, the number of points over these outlines will be proportional to their areas *Kuthan* (1968) used another modification of point counting technique for the assessment of the extent of aortic atherosclerosis, by means of multiple parallel cuts and cross cuts in the aortic intima he extended the examination also in depth, thus trying to improve the definition of the severity of atherosclerotic lesions

So far, the point counting technique has not been applied to the analysis of the extent of coronary atherosclerosis In the following we describe a modification of the point counting technique developed for that purpose Data on the reproducibility of the method are presented and the results obtained by it are compared with those obtained by planimetry By way of comparison, the accuracy of the quantitative assessment of aortic atherosclerosis by the original point counting technique of *Mitchell & Cranston* is presented, based on the same autopsy series

MATERIAL AND METHODS

Coronary Artery and Aortic Specimens

This methodological study was carried out in connection with a larger study project on coronary and aortic atherosclerosis based on a series of 559 cases autopsied at the Department of Forensic Medicine, University of Helsinki The specimens of coronary arteries and aorta for the assessment of atherosclerotic lesions were prepared according to the principles employed in recent extensive international studies (*Uemura et al* 1964, *Guzman et al* 1968) At autopsy the right coronary artery, the left anterior descending coronary artery and the left circumflex coronary artery were opened longitudinally with blunt pointed scissors The coronary arteries were dissected out as far as they could be cut open Two specimens were taken from the aorta—a segment of the thoracic aorta from the superior paired intercostal artery to the coeliac artery and a segment of the abdominal aorta from the coeliac artery to the

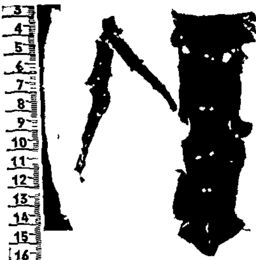


Fig 1 Specimens of the coronary arteries and the abdominal aorta

bifurcation The coronary and aortic specimens were flattened on cardboard, fixed in formalin and stained with Sudan IV solution (Fig 1)

Types of Atherosclerotic Lesions Studied

The areas of the following atherosclerotic lesions were measured from the Sudan-stained arterial specimens

Fatty streak a flat or slightly elevated intimal lesion that is stained distinctly by Sudan IV and does not show any other type of change underlying it

Fibrous plaque a firm, pale grey elevated intimal lesion, after staining it may be partly or completely covered with sudanophilic material, but does not exhibit ulceration, haemorrhage or thrombosis

Complicated lesion ulceration, mural haemorrhage or mural thrombosis

Raised lesion combination of fibrous plaque and complicated lesion

For the demonstration of *calcified lesions* the arterial specimens were radiographed on *Crystal lex®* film at a tube distance of 80 cm, other exposure factors being 50 kV, 15 ma, 1 sec. The arterial specimens were placed directly on the film envelop to prevent geometric enlargement

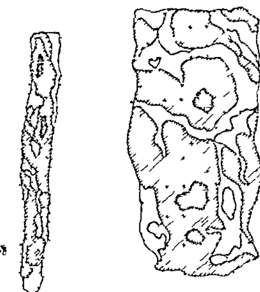
The Principles Employed in Point Counting

In the method used by *Mitchell & Cranston* for area measurements in the aorta the transparent sheet placed over the specimen has a point grid with the points 6 35 mm apart at the corners of equilateral triangles Since the error of this method is a function of the number of points counted

(*Dunnill* 1962, *Mitchell & Cranston* 1965) a distance of the points in the grid was sought for coronary artery measurements in which the number of points to be counted per coronary artery would be the same as those counted per aortic segment. Experimentally 2.0 mm was found to be a suitable distance between the points. Using this distance of points in the grid a total of over 600 points are counted in the point-counting measurement of three coronary arteries in the adult.

For measurement by point-counting the arterial specimen was placed firmly fastened between a stiff plastic sheet and the thin transparent plastic sheet with point grid. Fig. 2 shows a comparison of the modification of the point-counting technique for coronary arteries and the original technique of *Mitchell & Cranston* for the aorta. In each coronary artery and aortic segment a count was made of the number of points overlying areas of normal intima, fatty streaks, fibrous plaques and complicated lesions. The count for raised lesion was obtained by combining the number of points overlying the areas of fibrous plaques and complicated lesions. Since the number of points overlying the total area of the arterial specimen was simultaneously obtained the percentage areas for each type of the lesion could be calculated. The points falling over calcifications were counted from the radiographs (Fig. 3), using the same point grids as in the measurement from arterial specimens.

When the points in the grid have a regular arrangement, also absolute areas can be assessed with the point-counting technique. In the aorta the absolute areas can be calculated by multiplying the number of points counted for the measured area with the area represented by one point count which is 0.349 cm² in the method of *Mitchell & Cranston* (1965). In the point grid used in the present study for measurement of coronary arteries each point count accounts for an area of 0.0346 cm². In the following analysis of the reproducibility of the point-counting technique, point counts were not transformed to absolute areas.



normal intima

fatty streak

fibrous plaque

complicated lesion

Fig. 2. Point counting grid in the modified technique for the coronary arteries and point counting grid in the original technique of Mitchell and Cranston for the aorta overlying tracings of arterial specimens showing different types of atherosclerotic lesions.

Testing of the Reproducibility of Point Counting

The assessment of coronary atherosclerosis was done twice from arterial specimens obtained from 24 autopsied cases. In three cases an unusually large marginal branch of the left coronary artery was measured separately. Thus the total number of coronary arteries measured was 75. Replicate measurements were made also from the thoracic and abdominal segments of the aorta, totalling 48 segments from the same subjects. The first point counting measurement for each arterial specimen was performed in connection with the larger study project mentioned before. The second point count was done several months later in an unselected sample of stored arterial specimens. Both assessments were carried out by the same investigator.

The following estimates of the measurement error were calculated:

standard deviation of the random measurement error (S Δ) obtained from the formula

$$S\Delta = \sqrt{\frac{\sum_i (x_{1i} - x_{2i})^2}{2N}}$$

in which x_{1i} is the point count for sample i in the first measurement, x_{2i} is the point count for sample i in the second measurement and N is the number of observations.

S Δ as a percentage of the mean value of the replicate measurements (S %).

correlation coefficient of the first measurement on the second measurement.

Comparison of Point Counting and Planimetry

Planimetric measurements were made of 30 coronary arteries and 20 aortic segments from 10



Fig 3 Calcifications in radiographs of coronary artery and aortic specimens

subjects Using arterial specimens stored in plastic bags the contours of the opened artery and of the total affected area i e fatty streaks and raised lesions combined were traced on transparent tracing paper The affected area as percentage of the total area of the arterial specimen was measured by point counting from coronary and aortic tracings The planimetric measurement was carried out by weighing method The tracing of the total artery area was cut out and weighed, then the area affected by atherosclerotic lesions was cut out and weighed separately for the calculation of the percentage of the affected area from the total area

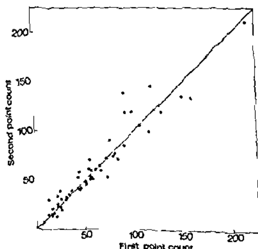


Fig 4 Comparison of the number of points counted in replicate assessments of the area of raised lesion in coronary arteries

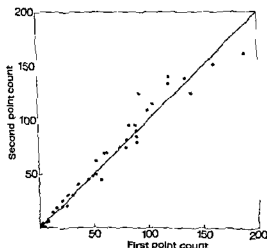


Fig 5 Comparison of the number of points counted in replicate assessments of the area of raised lesion in aortic segments

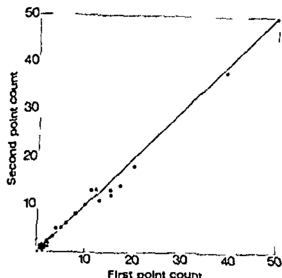


Fig 6 Comparison of the number of points counted in replicate assessments of the area of calcifications in radiographs of coronary arteries

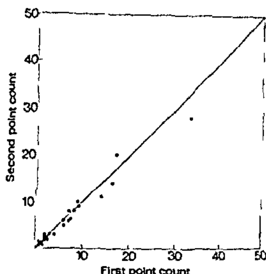


Fig 7 Comparison of the number of points counted in replicate assessments of the area of calcifications in radiographs of aortic segments

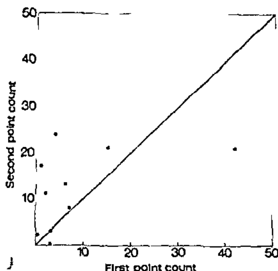


Fig 8 Comparison of the number of points counted in replicate assessments of the area of complicated lesion in coronary arteries

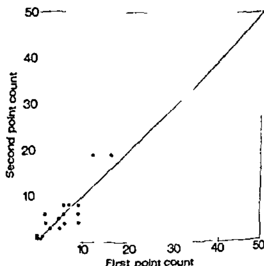


Fig 9 Comparison of the number of points counted in replicate assessments of the area of complicated lesion in aortic segments

RESULTS

Reproducibility of Results of Point Counting

Comparison of replicate point counts of the area of raised lesions calcifications and complicated lesions in the coronary arteries and in the aorta are presented in Figs 4–9 In Table 1 mean values and standard deviations are given for replicate point counts of the total area of coronary and aortic specimens and for the areas of each type of atherosclerotic lesions and the estimates of the method error of point counting are presented

The results indicate that the reproducibility of the assessment of the total coronary

TABLE 1 *Comparison of the Number of Points Counted in Replicate Determinations of the Area of the Artery and Various Types of Atherosclerotic Lesions in Coronary Arteries and Aortic Segments*

Variable	No of ob servations	First point count mean	First point count s d	Second point count mean	Second point count s d	S Δ*	S %°	$r_{1,2}$§
otal area of coronary artery	75	212 6	88 3	213 2	88 2	4 38	2 06	997
otal area of aortic segment	48	199 2	68 7	199 6	69 3	6 01	3 02	992
atty streak coronary artery	74	29 4	29 0	29 6	28 3	4 44	15 0	976
atty streak, aorta	48	44 0	24 8	42 5	22 9	4 99	11 6	960
aised lesion coronary artery	53	59 4	43 1	60 4	41 8	7 33	12 2	970
Raised lesion aorta	40	60 6	48 6	62 6	49 0	7 05	11 4	979
alcif cations coronary artery	29	6 86	8 48	6 52	8 01	0 81	12 1	993
alcifications aorta	27	6 52	7 14	6 19	6 41	1 05	16 5	982
Compl cated lesion coronary artery	10	8 30	12 6	12 0	8 65	7 99	78 7	489
Complicated lesion aorta	19	5 21	4 43	5 68	5 29	1 84	33 8	868

* S Δ – standard deviation of the random measurement error
° S % – S Δ as a percentage of the mean value of the replicate measurements
§ $r_{1,2}$ correlation coefficient of the first measurement on the second measurement

artery area using the point distance of 2 0 mm is as good as the reproducibility of the measurement of the total area of aortic seg ment using the original technique of Mitchell & Cranston in which the point distance is 6 35 mm

The method error in the area measurement of atherosclerotic lesions by the point count ing technique is a result of both the random scattering of points over the lesions and of the subjetivity of the definition of the out lines of the lesions The number of points counted per atherosclerotic area is, of course, smaller than the number of points overlying the total area of the arterial specimen As a consequence of the interdependence of the method error and the number of points counted, the error in the assessment of the area of atherosclerotic lesions is relatively greater than in the assessment of the total area of arterial specimen Nevertheless, the reproducibility of the area assessments for fatty streaks and raised lesions was reasonably good both in the coronary arteries and in the aorta In spite of the fact that the number of points counted in the assessment of calci fications was much smaller than that counted in the assessment of fatty streaks and raised lesions, the reproducibility of the assessment of calcifications was good both in the coro- nary arteries and in the aorta The S % for these types of lesions was approximately of the same magnitude both in the coronary arteries and in the aorta It must be taken into account that measurements of calcified areas were made from radiographs and their definition was more independent of the sub jective interpretation than the definition of other types of lesions made from arterial specimens The assessment of complicated lesions proved to be more poorly reproducible than the assessment of other types of athero- sclerotic lesions both in the coronary arteries and in the aorta The second assessment of

the coronary arteries tended to yield higher results than the first assessment

Accuracy of Point Counting in Comparison with Planimetry

Figs 10 and 11 show a comparison of the percentage areas of atherosclerotic lesions obtained by point counting made *from coronary* artery specimens and from tracings of these specimens with the percentage areas of lesions obtained by planimetry Mean values and standard deviations of percentage areas of the lesions in the coronary arteries and *in the* aortic segments assessed by three types of measurements i e point counting from arterial specimens point counting from tracings and planimetry, are given in Table 2 In addition the results of a comparison between the point counting assessment from arterial specimens and the assessment by planimetry and of a comparison between the point-counting assessment from tracings and the assessment by planimetry, carried out by regression analysis are given in Table 2

The accuracy of point counting carried out on clearly demarcated areas of coronary and aortic tracings was well comparable with that of planimetry A good correlation was observed also between the percentage areas of lesions assessed by point counting from arterial specimens and the corresponding areas assessed by planimetry

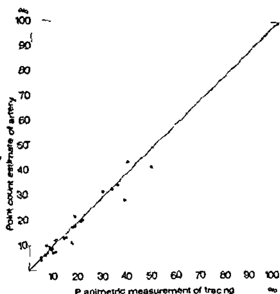


Fig 10 Comparison of point count estimates of the percentage surface area involved by atherosclerosis in coronary artery specimens with planimetric measurements of the percentage surface area representing atherosclerosis on tracings from the same specimens

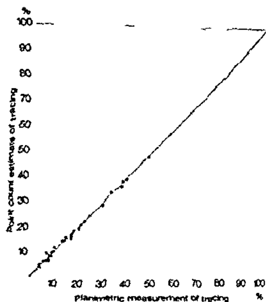


Fig 11 Comparison of point count estimates and planimetric measurements of the percentage surface area representing atherosclerosis on coronary artery tracings

COMMENT

The results presented indicate that provided the distance *between the points in* the grid is suitable the accuracy of the point counting method in the assessment of the extent of coronary atherosclerotic lesions is of the same order as the accuracy of the assessment of aortic atherosclerosis areas by the original technique of *Mitchell & Cranston* (1965) In principle the point counting technique can be applied to the quantitative assessment of atherosclerosis in any artery provided the point grid is modified so that the number of points to be counted is in suitable relation *to the area of the artery The results obtained with the point-counting technique* are well reproducible and comparable with those obtained by planimetry Point counting is how

TABLE 2 *A Comparison of Point Count Estimates of the Percentage Surface Area of Atherosclerotic Lesions Made from Coronary Artery and Aortic Specimens and from Tracings of the Same Specimens with Planimetric Measurements of the Same Parameter, Carried out by Means of Regression Analysis*

No of observations	Y	X	$mean_y$	$s d_y$	$mean_x$	$s d_x$	r_{xy}*	SEE°	Slope	Intercept
Coronary arteries										
30	point count of artery	planimetry of tracing	17 8	11 6	18 4	12 1	969	2 97	+0 924	+0 771
30	point count of tracing	planimetry of tracing	18 3	11 8	18 4	12 1	997	0 95	+0 971	+0 433
Aorta										
20	point count of artery	planimetry of tracing	22 9	16 1	21 0	15 4	971	4 07	+1 013	+1 613
20	point count of tracing	planimetry of tracing	21 6	15 2	21 0	15 4	997	1 24	+0 982	+0 899

* r_{xy} = correlation coefficient
° SEE = standard error of estimate

ever, more simple to perform and less time-consuming than planimetry In comparison with the visual assessment of percentage areas of atherosclerotic lesions the method is rather slow, measurement of three coronary arteries and two aortic segments requires about half an hour

In the point-counting method, as also in planimetry, the definition of atherosclerotic lesions depends ultimately on subjective judgment The definition of the outlines of fatty streaks is facilitated with Sudan staining The boundary between a fatty streak and a raised lesion however, is often difficult to define, as is also the boundary between a raised lesion and a diffusely thickened intima The definition of calcified lesions from arterial specimens is also difficult but they can readily be demonstrated in radiographs One of the advantages of point-counting is the possibility to assess calcified areas from radiographs with the same accuracy as other atherosclerotic lesions are assessed from arterial specimens On the other hand, the point-counting method does not take into consideration thickening of the wall and narrowing of the lumen in coronary arteries Cross cuts in the intima, as proposed by *Kuthan* (1968), may be of help in the definition of lesions in the aorta but they are probably not suitable for the coronary arteries

The reproducibility of area measurement of complicated lesions by point-counting was poor in comparison with that found for other types of lesions The second assessment of coronary arteries tended to yield higher values for this lesion This was obviously due to the fact that, in the first measurement, the compression applied to the specimen made the lesions more extensive Hence, the comparison of replicate point counts is, in part at least, misleading in regard to the reliability of the assessment of complicated lesion in the actual measurement situation where all the lesions are measured simultaneously

It is difficult to give definite anatomical landmarks up to which coronary artery specimens should be taken at their distal end Systematic differences in the total area of coronary artery specimens, arising from the technical point mentioned, lead to differences in the calculated percentage areas of atherosclerotic lesions since the lesions are generally located

in the proximal parts of the coronary arteries Therefore, the absolute areas of lesions may be better comparable than their percentage areas in studies made by different investigators With the point-counting technique the absolute or percentage areas of atherosclerotic lesions in three coronary arteries can be readily combined to express the absolute or percentage extent of atherosclerosis in the whole coronary arterial tree If desired, the areas of given types of atherosclerotic lesions can also be combined, for example for the calculation of the area of raised lesion

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This study was supported by grants from *Paavo Ilmari Ahtenainen Foundation* and from *The National Research Council for Medical Sciences in Finland*

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Acta path microbiol scand Section A 80 421–426 1972

DIFFERENCES IN ^{3}H-THYMIDINE UPTAKE BY CHICKEN LYMPHOID TISSUES AFTER INTRAVENOUS OR INTRAPERITONEAL ADMINISTRATION

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Intraperitoneal injection of tritiated thymidine, as compared to intravenous injection, was shown to cause a local labelling of the organs inside the peritoneal cavity This labelling was most intensive in organs situated in the part of the cavity where the injection was made In autoradiographs of ileum from the intraperitoneally labelled animals heavily labelled cells were found in the serous membrane the muscular layers, and even in the lamina propria close to the surface epithelium. It could be calculated that the tritiated thymidine available in the general circulation after an intraperitoneal injection was about 60 per cent of that available after an intravenous injection of the same dose

During the last 15 years tritium labelled thymidine (^{3}H Tdr) has been extensively used as a precursor of DNA in a variety of experiments in biological science Thymidine (Tdr) is rapidly and exclusively incorporated into DNA in most organisms, although Tdr itself is not essential for DNA synthesis and does not occur naturally in the main intra cellular metabolic pathways that lead to DNA synthesis (5) The incorporation of Tdr is possible however, after a single phosphorylation to Tdr monophosphate, which is a natural DNA precursor

When ^{3}H Tdr is used in experiments *in vivo* it can be administered in different ways For example, local administration directely into the thymus parenchyma has been used for local labelling of the thymic lymphocytes (10), and systemic administration has been used for labelling all cells in the body undergoing DNA synthesis (8) The commonly used methods for obtaining a systemic distribution are by injection of ^{3}H Tdr intravenously or intraperitoneally

It has been observed by several investigators (2 12) that the intraperitoneal injection of ^{3}H-Tdr, in contrast to intravenous injection, can cause local labelling of cells lining the peritoneal cavity Since the total uptake of ^{3}H-Tdr in the whole animal is the same wether it is administered intraperitoneally or intravenously (12), these results indicate that there are differences in the distribution of the labelled precursor when administered by these two routes

Since both intravenous and intraperitoneal administration are often used in experiments involving labelling with ^{3}H-Tdr *in vivo*, it seemed of great technical importance to study differences in the uptake of the ^{3}H-Tdr after these methods of administration The aim of the present investigation was therefore, to measure the incorporation of ^{3}H Tdr into different organs in the chicken after

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intravenous or intraperitoneal administration of the ^{3}H-Tdr

MATERIAL AND METHODS

Animals Twenty-eight 9 day-old male chickens of the non inbred strain Babcock B 300 were used The chickens were matched by weight into two groups with 14 animals in each, one group for intravenous and one for intraperitoneal labelling with ^{3}H Tdr The weight of the chickens at the beginning of the experiment was 61 ± 4 g (M ± SD)

Labelling The animals were labelled by a single injection of ^{3}H Tdr (spec act 6 7 Ci/mM New England Nuclear Corp, Boston Mass. USA) either intravenously into one of the wing veins or intraperitoneally through the abdominal wall The isotope dose (0 05 μCi/g body weight) was the same in all animals A solution containing 0 5 mCi of ^{3}H Tdr per ml sterile water was first diluted tenfold with sterile physiological saline to give a concentration of 0 05 mCi/ml A microlitre syringe (Hamilton Comp Inc, Whittier, Calif USA) with a fine gauge needle (external diameter 0 30 mm) was then used to administer the ^{3}H Tdr The same dilution of the isotope and the same type of syringe and needle were used in the two groups

Dissection procedure The animals were killed by decapitation 60 minutes after labelling The following organs were sampled the thymus the bursa of Fabricius bone marrow from the left femur, the spleen the caecal tonsils the liver, and a 2 cm piece of duodenum and ileum Samples from all organs were then used for DNA extraction and tritium measurements Autoradiographs were prepared from sections of ileum

Radiochemical studies The organ samples were put in 5 ml of 5 per cent trichloracetic acid (TCA) and immediately frozen until extraction of nucleic acids was performed using a modified *Schneider* (14) technique in which the lipid and phosphoprotein removing steps were omitted The organ samples were homogenized with a cold Potter Elvehjelm homogenizer centrifuged and washed twice with cold 5 per cent TCA The nucleic acids were then extracted with 5 ml of 5 per cent TCA in a water bath at + 90°C for 30 minutes and the DNA content of the supernatant was measured with *Burton's* modification (3) of the diphenyl amine reaction The radioactivity in 1 ml of the supernatant was measured in a Packard Tri Carb 3380 liquid scintillation counter at + 2°C using 10 ml of Insta Gel (Packard Instrument Comp Inc Warrenville, Ill USA) as scintillator One ml of 5 per cent TCA (without nucleic acids) was used for background correction The control of quenching was applied using the external standard channels ratio method (11)

Calculations In the results and discussion of the radiochemical studies the following terms are used specific activity (spec act.) expressed as counts per minute/mg DNA (cpm/mg DNA) and relative activity (rel act) defined as the spec act of an organ divided by the sum of the spec act of the thymus and bone marrow from the same animal

The spec act was calculated from the results of the determinations of DNA and tritium The rel act mean of rel act and standard error of the mean of rel act were calculated for all organ samples The statistical analyses were performed with *Student's* t test or when the standard error of the mean differed between the two groups with the approximation of the *Behrens Fisher* test according to *Cochran* (6)

The rel act, calculated as described above can be used for a comparison of the labelling of different organs with that of the thymus and the bone marrow the two organs sampled which should be labelled solely by ^{3}H Tdr available from the general circulation A significantly higher rel act of an organ in the intraperitoneally labelled than in the intravenously labelled group indicates that the cells of that organ in the former group have been labelled not only by the ^{3}H Tdr from the general circulation but also by ^{3}H Tdr from another local source

Autoradiographic technique Autoradiographs of sections of the ileum were prepared with the liquid-emulsion technique using NTB-2 emulsion (Eastman Kodak Comp, Rochester NY USA) For details see (4) The slides were exposed for 21 days in light proof boxes and subsequently developed in Kodak F 24 for 2 minutes and fixed in Kodak D 19b for 10 minutes The sections were stained through the emulsion with *Mayer's* haemalum

RESULTS

Radiochemical studies The specific activities of the dissected organs are recorded in Table I These figures show that the amount of available ^{3}H Tdr differed for the same organs in the intravenously and the intraperitoneally labelled animals In organs outside the peritoneal cavity the thymus and the bone marrow, which should be labelled solely by ^{3}H Tdr from the general circulation, the uptake of ^{3}H Tdr was significantly lower in the intraperitoneally labelled than in the intravenously labelled group ($p < 0.001$) In both

TABLE 1 *Means and Standard Error of the Means of Specific Activity* of Different Organs in 9 Day-Old Chickens Labelled Intravenously or Intraperitoneally with Tritiated Thymidine and Sacrificed 1 Hour Later*

Route of injection of ^3H-thymidine	Organ: Thymus	Bursa of Fabricius	Bone marrow	Spleen	Caecal tonsils	Liver	Duodenum	Ileum
Intravenously	1 900 ± 200	12 900 ± 700	33 700 ± 2 800	19 400 ± 1 500	13 900 ± 1 100	3 600 ± 300	16 500 ± 1 100	17 700 ± 2 100
Intraperitoneally	1 100 ± 100	9 700 ± 800	19 500 ± 1 800	13 200 ± 1 400	17 200 ± 2 300	3 700 ± 400	10 600 ± 700	19 800 ± 3 500
	$p < 0.001$	$p < 0.005$	$p < 0.001$	$p < 0.01$	----	----	$p < 0.001$	----

* Specific activity = cpm/mg DNA

TABLE 2 *Means and Standard Error of the Means of the Relative Activity* of Different Organs in 9 Day-Old Chickens Labelled Intravenously or Intraperitoneally with Tritiated Thymidine and Sacrificed 1 Hour Later.*

Route of injection of ^3H thymidine	Organ: Bursa of Fabricius	Spleen	Caecal tonsils	Liver	Duodenum	Ileum
Intravenously	0.45 ± 0.06	0.59 ± 0.01	0.48 ± 0.07	0.11 ± 0.01	0.51 ± 0.04	0.56 ± 0.06
Intraperitoneally	0.52 ± 0.07	0.68 ± 0.09	0.73 ± 0.10	0.20 ± 0.03	0.56 ± 0.07	1.11 ± 0.26
	----	----	$p < 0.05$	$p < 0.05$	----	$p < 0.05$

* Relative activity is defined as specific activity of an organ divided by the sum of the specific activities of the thymus and the bone marrow from the same animal

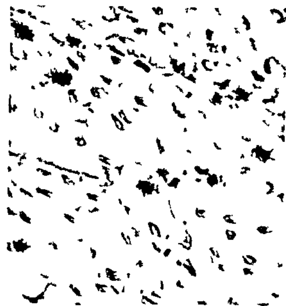


Fig 1 Locally labelled cells in the muscular layer of the small intestine (ileum) after intraperitoneal administration of 0.05 μCi/g body weight of ³H thymidine (Autoradiograph 1000 × Haemalum)

organs the uptake of ³H Tdr in the intraperitoneally labelled group was 58 per cent of the uptake in the intravenously labelled group The spec act also show that the uptake of ³H Tdr was low in the thymus and high in the bone marrow compared to other organs

Organs inside the peritoneal cavity showed a different labelling pattern As regards the bursa of Fabricius the spleen and the duodenum samples the spec act was lower in the intraperitoneally labelled animals although the differences were not as pronounced as for the thymus and the bone marrow In contrast, the means of the spec act of the ileum the caecal tonsils and the liver were higher in the intraperitoneally labelled than in the intravenously labelled animals although the differences did not reach the level of statistical significance

From the rel act shown in Table 2 it can be seen that the values for the ileum the caecal tonsils and the liver were significantly higher ($p < 0.05$) in the intraperitoneally labelled group indicating that these organs were labelled not only by ³H Tdr from the general circulation but also by ³H Tdr from another local source The rel act of the bursa of Fabricius the spleen and the duodenum did not differ significantly between the two groups indicating that these organs were labelled mainly by ³H Tdr available from the general circulation A certain degree of additional local labelling of these organs could not however be excluded

Autoradiography When examining the autoradiographs of sections of ileum from the intravenously labelled animals no cell with grain counts greater than 15 could be found In sections from animals labelled intraperitoneally, cells with very heavy labelling (30 grains and more) over their nuclei were easily found especially in the serous membrane and the muscular layers but also in the lamina propria under the surface epithelium (Fig 1) The frequency of such heavily labelled cells varied from one animal to another

DISCUSSION

A rapid distribution throughout the body is important when ³H Tdr is used as tracer since Tdr is quickly removed from the circulation and incorporated by proliferating cells or catabolized The removal of ³H Tdr from the blood stream after intravenous or intraperitoneal injection has been shown to be almost identical One hour after administration 98–100 per cent of intravenously injected (15) and 96 per cent of intraperitoneally injected thymidine is absorbed (13) Since ³H Tdr injected intraperitoneally causes a greater labelling of cells in organs inside the peritoneal cavity than after intravenous injection (12) it appears that there is an unequal distribution of intraperitoneally administered label between organs inside and outside the peritoneal cavity The present investigation was therefore designed to measure the uptake of ³H Tdr by different organs in animals which had been injected intravenously or intraperitoneally with the same dose of isotope

The results clearly show that the route of

administration has a considerable effect on the incorporation of tritiated thymidine into cells of different organs in the chicken The values for the spec. act of the organs sampled confirm the findings of other investigators (7, 16) that the uptake of ^{3}H-Tdr in the thymus is low, even though the thymus has a high rate of DNA synthesis (1, 7) The explanation of this is not quite clear Bone marrow, however, has a very high uptake of ^{3}H Tdr which in this organ correlates with a high rate of DNA synthesis These two organs are not in the peritoneal cavity and should therefore be labelled solely by ^{3}H Tdr from the blood circulation When comparing their spec act between the intravenously and the intraperitoneally labelled groups it can be calculated that the ^{3}H Tdr available in the general circulation after an intraperitoneal injection is about 60 per cent of that available after an intravenous injection of the same dose This may be because intraperitoneally injected ^{3}H Tdr can be locally incorporated into cells lining the peritoneal cavity, or that the ^{3}H Tdr can be catabolized in the liver, since most thymidine absorbed intraperitoneally has to pass through the liver to reach the general circulation However, *Petersen* & *Baserga* (12) showed that the total uptake of ^{3}H Tdr was the same whether injected intra venously or intraperitoneally These results suggest that a local uptake by cells in the peritoneal cavity may be the main reason why less ^{3}H Tdr is available in the general circulation after an intraperitoneal injection This interpretation is supported by the values of the spec act *for organs in the peritoneal* cavity determined in the studies reported here

From the rel act it can be concluded that the ileum the caecal tonsils and the liver in the intraperitoneally injected animals were labelled significantly by locally available ^{3}H-Tdr It could not be excluded that some local labelling also occurred in the other organs located in the peritoneal cavity, although the incorporation of label was not significantly higher than in the intravenously labelled animals The local labelling of the ileum was verified by autoradiographs, where cells with very heavy labelling were easily found, al though the dose of isotope used in this experiment was kept rather low, ten times lower than that commonly used (5, 7, 9) These heavily labelled cells were most frequent in the serous membrane and the outer parts of the muscular layers, but heavily label led cells could even be found in the lamina propria close to the surface epithelium The local labelling of the ileum and the caecal tonsils is explicable in that these organs are situated in the part of the peritoneal cavity where the injected ^{3}H Tdr was deposited and consequently where the concentration was highest The local labelling of the liver in the intraperitoneally labelled group may be the sum effect of labelling by diffusion of ^{3}H Tdr from the peritoneal cavity, and labelling by ^{3}H Tdr resorbed intraperitoneally and transported to the liver via the portal vein

Thus, the present investigation shows that intraperitoneal injection of ^{3}H Tdr causes a local labelling of the organs in the peritoneal cavity and that this labelling is most intensive in organs situated in parts of the cavity where the injection is made It was also shown that the ^{3}H-Tdr available in the general circula tion after an intraperitoneal injection was about 60 per cent of that available after an intravenous injection

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BRIEF REPORTS

SPLENIC INHIBITION OF THYMIC RELEASE OF LYMPHOCYTES

Ulf Ernstrom

Splenic humoral factors causing depression of blood cell counts in different laboratory animals are frequently reported in the literature and are hypothetically related to the human clinical syndrome called hypersplenism characterized by splenomegaly and deficiency in erythrocytes leucocytes or thrombocytes (1)

The present investigation was originally aimed to study the effect of a thymic hormone called thymosin (2) on the thymic release of lymphocytes Two groups of animals were used as controls animals treated with saline and animals treated with a splenic extract The results obtained indicate an inhibitory effect of the splenic extract on the thymic release of lymphocytes as will be reported here

A total of 71 male guinea pigs weighing 200–230 g were used They were divided into three groups which were treated with saline a thymic extract and a splenic extract, respectively

The thymic and splenic preparations were partly purified saline extracts from calf spleen and calf thymus (3) and were generously supplied by Dr *Goldstein* and Dr *White* The preparations obtained were dissolved in physiological saline to a concentration of 60 mg per ml. The solutions were then passed through a Millipore filter for the purpose of removing insoluble particulate material and for sterilization purposes The experimental animals were given a single subcutaneous injection in the dorsum with 0 2 ml of the thymic, the splenic or the saline solution All animals were investigated 6 hrs after the treatment

In a first experiment in which 27 animals were utilized the thymic blood flow was determined by the method of *Larsson* (4) The blood flow through one thymic lobe was registered in μl per min

In a second experiment including 44 animals, the number of lymphocytes per μl of blood from the thymic vein and the carotid artery was determined according to *Ernstrom* & *Larsson* (5) The thymic veno-arterial difference in number of lymphocytes was obtained from each animal The differences were analysed statistically by Student s t test

The thymic release of lymphocytes into the blood per min was calculated from the thymic blood flow and the thymic veno-arterial difference in number of lymphocytes The standard deviation of the product was obtained from the formula

$$S_{xy} = \sqrt{x^2 \cdot S_y^2 + \bar{y}^2 \cdot S_x^2}$$

where x = mean blood flow, S_x = standard deviation of x $\bar{y}$ = mean veno-arterial difference S_y = standard deviation of y

The results are seen in Table 1 The thymic blood flow was not significantly changed by either treatment The thymic veno arterial difference in number of lymphocytes per μl of blood passing through the thymus was statistically significant in the animals treated with the thymic preparation and with saline, but not in animals treated with the splenic preparation The results thus indicate a net release of lymphocytes from the thymus in the animals treated with saline and in those treated with the thymic extract but not in those treated with the splenic extract

The thymic veno-arterial difference in number of lymphocytes per μl of blood after treatment with the thymic extract was not significantly different from that in the animals treated with saline The difference after treatment with the splenic extract was significantly different from that obtained after treatment with the thymic extract ($p < 0.01$) This indicates a factor in the splenic extract inhibiting the net release of lymphocytes from the thymus

A difference in cellular content in thymic afferent and efferent blood must be due to the contribution of thymic cells to the efferent blood Of course such a difference must be influenced by a possible uptake of cells in the thymus from the afferent blood Thus, an abolished veno arterial difference may be caused either by an inhibited release of lymphocytes from the thymus or by an increased trapping of lymphocytes in the thymus from the afferent blood The present results cannot discriminate between these two possibilities

Received 20 iii 72 from the *Department of Histology Karolinska Institutet 10401 Stockholm 60 Sweden*

TABLE 1 *Calculation of the Thymic Release of Lymphocytes per min from the Thymic Blood Flow and the Thymic Veno Arterial Difference in Number of Lymphocytes per µl of Blood*

	Thymic vein blood (lymphoc./µl)	Carotid artery blood (lymphoc./µl)	Veno-arterial difference (lymphoc./µl)	Thymic blood flow (µl/min)	Thymic release (lymphoc./min)
Saline	2832±355 (14)	2252±252 (14)	580±258 $p < 0.05$	44.2±9.1 (9)	25636±12558 $p < 0.05$
Thymic extract	2568±223 (16)	1906±150 (16)	662±165 $p < 0.001$	47.9±5.7 (8)	31710±8753 $p < 0.001$
Splenic extract	2822±241 (14)	2725±275 (14)	97±228	47.3±11.7 (10)	4588±10844

Three groups of animals treated with saline, a thymic extract or a splenic extract are compared. All figures are given for one thymic lobe. Number of animals within brackets. Mean ± SE.

Quantitatively, the inhibitory effect of the splenic extract on the net release of lymphocytes from the thymus is comparable to that of whole body irradiation of guinea pigs with 150 rad (6). It cannot be concluded from the present study whether the splenic factor is normally secreted from the spleen, whether it is of physiological or pathological importance or even whether it consists of one single chemical compound. However, the present result is compatible with the finding that an increased thymic release of lymphocytes occurs early after splenectomy (7) and thus might have been due to the withdrawal of a normal hormonal inhibition exerted by the spleen on the thymic release of lymphocytes.

This investigation was supported by a grant from the *Swedish Cancer Society*.

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Acta path. microbiol. scand. Section A, 80: 428–429, 1972.

THE ORIGIN OF DOUBLE MINUTES IN A ROUS RAT SARCOMA

Felix Mitelman, Goran Levan and Joachim Mark

The origin and nature of the remarkable chromosomal deviation called double minutes (dms) by *Mark* (1967) is still a matter of debate (*Levan et al.* 1968, *Mark & Granberg* 1970). The very small size of the dms, often close to the border of visibility, has rendered difficult the understanding of their structure. Thus an ordinary centromere has been observed in them only exceptionally. One difficulty has been that in most cases it has been impossible to observe their first appearance in a material and thus to associate them to any particular cytogenetic event. Below we report on a Rous rat sarcoma in which the loss of three specific chromosomes seemed to be correlated to the appearance of the same number of dms.

Received 21.iii.72 from the *Institute of Pathology and Institute of Genetics, University of Lund, Lund, Sweden.*

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Material and methods

The primary sarcoma was induced in a female rat of the W/Fu inbred strain by the Schmidt Ruppin strain of the Rous sarcoma virus (RSV-SR), as described by *Mitelman* (1971) Several clones were isolated from a primary culture of this tumour (*Levan et al* 1971), one of them (clone No 8) consisting of completely normal diploid cells This clone was re implanted into 4 rats of the same strain, and all inoculates grew progressively The 4 tumours resulting were subjected to chromosome analysis 16 to 21 days after inoculation, the chromosomes being studied in fixations directly from the tumours as described in *Mitelman* & *Mark* (1970) Karyotype analyses were made by photography

Observations and conclusions

In 3 of the sarcomas only normal diploid cells were observed as in the primary culture from which they were derived In each of them chromosome numbers were counted in 50 cells and karyotype analyses performed in 10 cells, all showing normal number and normal karyotype

In the fourth sarcoma 50 cells were karyotyped and they all fell into 2 classes (1) 9 cells with a perfectly normal diploid karyotype, and (2) 41 cells, among which 39 were hypodiploid and included 36 stemline cells with 2n 39 Two cells were hypotetraploid double stemline cells with 2n = 78 All these 41 cells, in addition to the normal chromosomes had 2 or more dms the hypodiploid cells had 2–4, usually 3 the hypotetraploid cells had 6 dms All hypodiploid cells had lost 3 m chromosomes and the hypotetraploid had lost 6 These facts made it tempting to conclude that the dms represented the centromeric regions of the 3 m chromosomes persistently lacking from the cells with dms Evidently the new stemline with dms was on its way to supress the normal diploid stemline which was in complete control in the 3 sister tumours In our opinion this means that the dms are capable of behaving as chromosomes at mitosis, even though their variability in number indicated a certain degree of mitotic failure Still it seems very likely that they had functioning centromeres As seen in Fig 1, each dm consisted of 2 chromatids The dms differed in size one was clearly bigger than the other 2 which were too small to display any reliable structures At least the biggest dm seemed to have a centromeric structure evidently located terminally, the small ones just showed a thread like connection between the chromatids At metaphase the dms were distribut

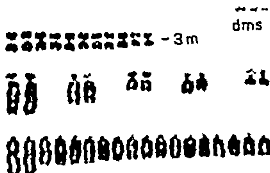


Fig 1 Stemline karyotype, 2n = 39 + 3 dms

ed at random among the regular chromosomes which may not mean very much in squash preparations At least they did not tend to appear in clusters, which would be expected if they were gathering into a specific nucleus of their own They kept their individuality, their variability in number being due rather to nondisjunction than to repeated events of fragmentation. The incidence of chromosome breakage was low

As stated already, the dms in the present case probably represent the centrometric regions of the 3 m chromosomes lost This conclusion gained support mainly from the biggest dm, in which a centromere like structure was sometimes clear A similar origin of a dm was assumed by one of us (*Mark* 1967) in a RSV SR induced mouse sarcoma (CBA283) in which one centromere was lost at the same time as one dm started to appear in the population In this tumour, the number of dms increased later on, and it is usual in materials with dms that there is a wide variation in their number and that often several are present The low and fairly constant number of dms in the present tumour may be a sign that the deviating stemline was of recent origin

This work was supported by grants from the *Swedish Cancer Society* and from *John and Augusta Persson s Foundation for Medical Research*

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting Stockholm November 26-27, 1971

C A Rubio & G Soderberg FACTORS INFLUENCING THE DETECTION OF CARCINOMA IN SITU IN CONE SPECIMENS

Reports in the literature indicate that the per centage of carcinomas in situ confirmed at coniza tion following punch or wedge biopsies varies from 62 to 97 per cent Some of the possible factors responsible for that wide variation have been ana lysed in 391 cone specimens The results demon strated that both the number and the size of the biopsies prior to conization may influence the pre sence of carcinoma in situ in cone specimens fewer lesions are found in cones preceded by larger or by multiple biopsies The results moreover demon strated that the size of the cone did not influence the presence of carcinoma in situ in such specimens similar percentages of carcinoma in situ lesions were found both in smaller as well as in larger cones

The number of sections made from each cone in fluenced the detection of carcinoma in situ 25 per cent new cases were detected by increasing the number of sections in negative cone specimens from 40 to more than 100 sections

The study of the histological type of the lesions demonstrated that cases with carcinoma in situ with buds were often associated with a higher proportion of cones with carcinoma in situ despite larger or multiple biopsies prior to conization

I Boquist B Lindqvist Y Östberg and L Steen MORPHOLOGIC STUDIES IN A CASE OF PRIMARY OXALOSIS

A man with primary oxalosis had a familial history of the disease and onset of symptoms in adult age The most important symptoms were progressive renal failure nephrolithiasis polyneuropathy and ischaemic alterations in the lower extremities lead ing to atrophy and gangrene He died in uraemia

The following morphologic methods were used light polarization, transmission electron and scanning electron microscopy Deposition of cal cium oxalate was found in kidneys various vessels heart striated muscles prostate and bones Other findings were chronic pyelonephritis, hydronephros is nephro- and ureterolithiasis old myocardial in farction atrophy of striated muscles and degenera tion of peripheral nerves The crystalline material in the kidneys was mainly localized to the epi thelium and the lumina of the tubules but was also found in glomeruli vessels and interstitium The crystals were composed of needle-shaped subunits It is believed that the oxalate crystals are formed intracellularly and that they secondarily are de posited extracellularly

G Unge & A Ljungqvist THE PROLIFERATIVE ACTIVITY IN EXPERIMENTAL CARDIAC HYPERTROPHY

In an earlier micro-angiographical investigation of the myocardial vascular reaction in various types of cardiac hypertrophy in rats we found features suggestive of a formation of new capillaries espe cially in swimtrained induced cardiac hypertrophy

To study this vascular reaction further the DNA synthesis in capillary wall cells fibroblasts and muscle cells in experimental cardiac hypertrophy in rats have been studied by autoradiographic tech nique after in vivo injections of H^3-thymidine Cardiac hypertrophy was induced by (i) hyper tension secondary to unilateral renal artery stenosis (duration one week and three months) (ii) aortic descendens stenosis (duration two months) (iii) swimtraining (duration three months) and (iv) swimtraining followed by rest three months train ing + two months rest)

The investigation has shown a clear increase of H^3-thymidine incorporation in capillary wall cells in the left ventricle wall of swimtrained rats This increase was not observed in the group that rested after swimtraining Also in rats with long term hypertension (three months duration) there was an increase in labelled capillary wall cells but this

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was of a minor degree A very low number of labelled muscle cells was noticed being most numerous in the swimtrained group

The observations suggest an increased proliferative activity in capillary wall cells, indicative of a formation of new capillaries in cardiac hypertrophy induced by swimtraining, and to a certain degree in long term hypertension The proliferative activity appears to decrease after termination of swim training

Edith Farkas, Krister Kristensson & Patrick Souranđer ENZYMEHISTOCHEMICAL CHANGES AT AN EARLY STAGE OF EXPERIMENTAL HERPES SIMPLEX ENCEPHALITIS

According to current opinion Herpes simplex encephalitis (HSE) is the most important of the sporadic viral encephalitides An excessive oedema of unknown origin is one of its main characteristics Since vascular walls and astrocytes are involved in the development of various types of cerebral oedema it was considered appropriate to use sensitive enzymehistochemical methods for detection of early changes in endothelial cells pericytes and astrocytes in experimental HSE

Mice 21 days old were inoculated intracerebrally with Herpes simplex virus The distribution in the brain of alkaline phosphatases of endothelial cells, acid phosphatases of pericytes and inosindiphosphatase of astrocytes was studied 2, 3, 4 and 5 days after inoculation by enzyme histochemical methods For studies of vascular permeability intravenously injected fluorescent tracers bound to protein were used

On the fifth day after inoculation when the clinical symptoms of HSE were full blown, increased vascular permeability for fluorescent protein indicating severe endothelial lesions was observed in both hippocampal regions In the same regions extensive necroses were present The endothelial cells displayed loss of activity for alkaline phosphatases and the pericytes reduced activity for acid phosphatases already on the fourth day after inoculation The changes were generalized and not confirmed to morphologically damaged regions Activity for inosindiphosphatase was strongly increased in the perikaryon and processes of astrocytes not only in morphologically affected regions but also in intact areas particularly in the cerebral cortex The generalized astrocytic changes appeared already on the third day after inoculation

In experimental HSE in mice the severe morphological destruction is mainly confined to hippocampal regions while enzyme histochemical changes i e reduced alkaline and acid phosphatase activities and increased inosindiphosphatase activity constitute a generalized phenomenon

Krister Kristensson & Yngve Olsson UPTAKE OF EXOGENOUS AGENTS IN OLFACTORY NERVE CELLS

A common mode of transmission of viral infections between various individuals is spread of nasal secretions through the air Certain neurovirulent viruses, e g, herpes simplex and arboviruses, may then spread from the nasal cavity of the recipient to the brain producing encephalitis The mechanism for such a virus transfer is unclear, but the possibility exists that the infectious agents may be taken up by the apical dendrites of the olfactory nerve cells, followed by an intra axonal transport to the brain In the present study we examined whether certain macromolecular substances can be taken up by these cells and whether the substances can spread to the olfactory brain after instillation into the nasal cavities

As tracing of viral particles in tissues is difficult to perform we used two different protein tracers which can be directly localized at a cellular level Albumin labelled with Evans blue and horseradish peroxidase were instilled into the nasal cavities of 12 day-old mice and at varying time intervals there after these tracers were localized to the olfactory mucosa, fila olfactoria and bulbus olfactorius by fluorescence and electronmicroscopy

Both substances spread throughout the nasal cavities, penetrated the mucos secretions and came into direct contact with the bulbous endings of the apical dendrites of the nerve cells They accumulated in pinocytotic vesicles of the nerve cell bodies and 1–24 hrs later they were found in fila olfactoria, bulbous olfactorius and the surrounding leptomeninges

Thus the present study shows that the bipolar nerve cells in the olfactory mucosa can take up macromolecules by pinocytosis from the nasal cavity and that a transfer of such substances into the olfactory brain may follow nasal instillations

N Forsby, U Brunk, J Ericsson, J Pontén & B Westermark ULTRASTRUCTURAL FEATURES OF IN VITRO CULTIVATED MALIGNANT GLIOMAS

The fine structure of *in vitro* cultivated human malignant glioma cells has been compared with that of glia like cells from normal, adult brain tissue The cells deriving from malignant gliomas differed from normal glia like cells in the following main respects

(*a*) The malignant cells formed abundant micro villi and extensive blunt cytoplasmic extrusions—even when growing in dense cultures The extrusions were sometimes found to be detached and appeared as regressively changed fragments of cytoplasm bordered by a membrane and containing

ground cytoplasm along with occasional organelles including lysosomes

(*b*) As a rule, the malignant cells lacked obvious specialization of different parts of their plasma membrane

(*c*) The malignant cells contained numerous autophagic vacuoles

These observations are compatible with greatly enhanced capability for plasma membrane motility, insufficient propensity for local surface specialization, inability to form tight and stable connections between adjacent cells and *increased turnover of* cytoplasmic constituents in the malignant—as compared to the non neoplastic—cells Repeated detachment of fragments of cytoplasm from malignant cells may result in considerable 'excretion' of lysosomal enzymes from these cells

Lennart C Eriksson MICROSOMES WITH FEW RIBOSOMES

A simple and effective method by which to isolate ribosome poor microsomal vesicles was worked out *Centrifugation* of mitochondrial supernate on a discontinuous CsCl-containing sucrose gradient at *102,000* g for *90* min gives a pellet (R_I microsomes), a *fluffy layer above the pellet* (R_{II} microsomes), and a vesicle fraction in the intermediate 1 3 M sucrose phase which is moderately influenced by CsCl (*R_{III} microsomes*) *The vesicles in the* interphase fraction are smooth microsomes The fractionation was performed in a 40 2 rotor (*Spinco-Beckman*) *The individual fractions were* removed by appropriate needles connected to a syringe The different membranes were chemically *and enzymatically analysed and their biosynthesis* was studied with radioactive precursors

After centrifugation of the different components, the distribution of protein was 49 8 15 and 28 per cent for R_I R_{II} R_{III} and Smooth respectively The protein/PLP ratio was almost the same for the four fractions after removal of adsorbed and content protein The RNA/PLP ratio decreased from R_I to Sm in agreement with the electromicroscopical pictures Enzymatically the R_{III} fraction shows the highest capacity with regard to the hydroxylation of drugs The specific activity of NADPH cytochrome c reductase exceeds the activity of the other fractions by almost 50 per cent NADH cytochrome c reductase cytochrome P 450, and cytochrome b_5 are also enriched in this microsomal fraction but to a lesser extent On the *other hand* R_{III} is characterized by low UDPGA transferase and phosphatase activities Studies of the incorporation of ^{3}H glycerol and ^{14}C-*leucine in* lipids and proteins, respectively, as well as of the one minute incorporation of ^{14}C leucine in ribosome adherent peptides show no *significant differences* between the rough fractions.

G Dallner TRANSPORT OF MOLECULES THROUGH MICROSOMAL MEMBRANES

Changes in microsomal membrane permeability influence the activity of many enzyme systems The passive permeability of microsomal membranes was investigated by utilizing radioactive substances and ultracentrifugation Unchanged substances up to a molecular weight of 600 penetrate freely On the other hand, the membrane exhibits a complete impermeability to charged substances. Treatment of microsomes with a hypotonic medium results in a free penetration of charged substrates to the intra microsomal compartment but not to specific enzyme sites within the membranes Such a condition is present *in alloxan diabetes After EDTA treatment* the same substances have free access to specific enzymes in different regions of the membrane This *unlimited penetration takes place after* administration of carbon tetrachloride Translocases of a protein nature seem to be absent in microsomes but *the polyprenol dolichol monophosphate* (DMP) known as a carrier lipid is present in smooth I and II and at especially high concentration in rough *microsomes* In the latter subfraction the concentration is of the same order as in the nuclear and Golgi fractions The transfer of glucose from the activated form of sugar to DMP is highest in smooth I microsomes Similar high transfer activity occurs only in the outer mitochondrial membranes Only rough and smooth I but not *smooth II microsomes* can utilize the glucosylated form of DMP as substrate in further reactions In this respect microsomes are unique since disregarding the moderate activity of plasma membranes no *other subcellular* particles can perform this reaction Microsomes, furthermore mediate interaction of ATP with microsomal DMP and DMP also stimulates *ATP* incorporation into the residual protein

Acta path microbiol scand Section A, 80, 433-439, 1972

A SENSITIVE IMMUNOFLUORESCENCE TECHNIQUE FOR DETECTING BLOOD GROUP SUBSTANCES A AND B

Findings in Oral Epithelium

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In order to select a sensitive method for demonstrating blood group substances A and B in sections of oral epithelium, biopsies and exfoliated cells from the oral epithelium were investigated with both a double layer immunofluorescence (IF) staining method and the mixed cell agglutination (MCA) reaction Eighteen anti A and 16 anti B sera were tested Fourteen anti A sera were able to react in the IF method and only 9 in the MCA reaction All tested anti B sera produced positive IF staining, eleven also reacted in the MCA reaction Blood group antisera could in most cases be used in higher titres in the IF method than in the MCA technique In the IF method, porcine antihuman IgG/FITC and porcine antihuman IgM/FITC were used as the second layers Blood group antisera could generally, but not invariably, be used in higher titres with antihuman IgM/FITC *The importance of careful matching of blood group antiserum and conjugate is emphasized*

The presence of blood group substances A and B in cells and tissues other than the erythrocytes was first demonstrated (11, 12) through the use of an agglutination inhibition test

In the oral mucosa, the presence of blood *group substances was first shown by absorp*tion of isoagglutinins in water-soluble extracts of oral epithelium (21)

These findings were later confirmed by the use of immunofluorescent (IF) and mixed cell agglutination (MCA) methods (6, 7, 17, 18) In contrast to the methods used previously, both of these techniques permit topographical study of blood group substances throughout the tissues of the body, including those on the cell membranes of the oral epithelium (17)

The MCA reaction on formalin-fixed paraffine embedded tissue is reported to be *more sensitive than the IF technique (4)* However, the IF technique will give more detailed information about the localization of the blood group substances Therefore, the objective of this work was to investigate methods by which the sensitivity of the IF technique for detection of blood group substances may be improved

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MATERIALS AND METHODS

The IF technique used in this study was a double layer fluorescence staining method (19) used on formalin fixed paraffin embedded tissue The first

layer was a human blood group antiserum, the second layer was a porcine antihuman globulin conjugated with fluorescein isothiocyanate (FITC)

The sensitivity of the technique was defined as the highest dilution of the first layer (blood group antiserum) giving a positive reaction when the second layer (the conjugate) was used in a dilution which was less than the plateau endpoint (20) Improvements in the sensitivity of the IF method were tried by changing the first and the second layer in the staining reaction The first layer was changed by using different blood group antisera in various dilutions the second layer by using conjugates to various human immunoglobulin classes

Improvements of the IF method were judged against the sensitivity of the MCA reaction, performed with unfixed exfoliated cells, as a base line The sensitivity of the MCA reaction was defined as the highest dilution of blood group antisera giving a positive reaction

Blood Group Substances

The A and B antigens used throughout were obtained from exfoliated epithelial cells and biopsies from normal buccal mucosa of two 25 year-old males Both were non-secretors in order to avoid contamination of cells and tissue with group substances from saliva one belonged to blood group A_1, and the other to blood group B

Blood grouping of cell and tissue donors was determined at the Blood Grouping Department Statens Seruminstitut, and their secretor status at the Department of Serology University Institute of Forensic Medicine, Copenhagen

Exfoliated cells were obtained by scraping the buccal mucosa with a wooden tongue depressor Incision biopsies were obtained from the buccal mucosa Local infiltration anaesthesia (2 per cent lidocain noradrenaline) was used and care was taken not to inject directly into the site of biopsy The biopsies were fixed for 12 hours at 20° C in 10 per cent neutral formalin freshly prepared from paraformaldehyde embedded in paraffin wax and sectioned at 5 µm.

Blood Group Antisera

The anti A and anti B sera were test sera provided by the Blood Grouping Department Statens Seruminstitut, and by the Department of Serology University Institute of Forensic Medicine Copenhagen

All sera were inactivated at 56° C for 30 minutes and stored at —20° C Serum dilutions 1 1 1 5 1 10, 1 20 1 40 were used

Conjugates

The conjugates used were porcine antihuman IgM/FITC globulin (SwAH IgM/FITC)® with a F/P ratio of 11,8 µg/mg and porcine antihuman IgG/FITC globulin (SwAH IgG/FITC)® with a F/P ratio of 15,0 µg/mg (Nordic Pharmaceuticals and Diagnostics, Tilburg, Holland) By chess board titration (20) a working titre of 1 20 was found for both conjugates. This conjugate titre was used throughout to allow comparison between titres of antisera

IF Staining and Fluorescence Microscope

The IF staining method was performed on 5 µm paraffin sections After dewaxing, the slides were incubated at room temperature in a moist chamber with dilutions of anti A (for blood group A subjects) or anti B (for blood group B subjects) sera for 20 minutes, washed three times for 5 minutes in phosphate buffered saline (P B S) at pH 7 2 and incubated for a further 20 minutes with conjugate Before mounting, the slides were washed again three times for 5 minutes and mounted in P B S with 10 per cent glycerol Two sections were stained with each blood group antiserum concentration in one of these the second layer was SwAH IgG/FITC®, and in the other the second layer was SwAH IgM/FITC®

Results were read as positive or negative with no attempt to grade the positive reactions The results were based upon readings in the stratum spinosum.

The fluorescence microscope was a Leitz Ortoplan® modified with a Tiyoda® wide-angle dark field oil immersion condenser The light source was an Osram HBO 200 lamp Magnification was 250 ×, using a 25 × plane fluorite objective NA 0 5 and periplan eyepiece The primary filter was a FITC interference filter with red contrast band (14, 15) (Laboratory for Technical Optics Lyngby, Denmark) The secondary filter was a 2 mm glass filter (Schott & Gen Mainz, Germany) matched to fit the primary filter (14, 15)

MCA Reaction

The MCA reactions were performed with the exfoliated cells as described previously (1) The principle of this technique is that bivalent or multivalent antibodies can combine with the antigen on the surface of the cells under investigation and at the same time with erythrocytes of known antigenic composition The results were read as positive or negative All cells with more than 5 detectable erythrocytes attached were regarded reactive and the MCA reaction was said to be positive when 10 out of 100 epithelial cells were registered as reactive

Control Reactions

To ensure that the fluorescence staining method and the mixed cell agglutination reactions were specific, the control reactions were made for both A and B blood groups These are summarized in

TABLE 1 *Controls for Establishing Specificity of Immunofluorescence Staining Controls for Anti A Test Sera*

Blood group substance	Blood group antisera	Conjugate	Results
A	Phosphate buffered saline	Labelled antiglobulin	Negative
A	Anti B	Labelled antiglobulin	Negative
A	Anti A absorbed with A_1 erythrocytes	Labelled antiglobulin	Negative
B	Anti A with known reactivity	Labelled antiglobulin	Negative
A	Anti A with known reactivity	Labelled antiglobulin	Positive

Analogous control reactions were made for anti B test sera

TABLE 2 *Controls for Establishing Specificity of Mixed Cell Agglutination Reaction Controls for Anti A Test Sera*

Blood group substance	Blood group antisera	Erythrocytes	Results
A	Phosphate buffered saline	A	Negative
A	Anti B	A	Negative
A	Anti A absorbed with A_1 erythrocytes	A	Negative
A	Anti A with known reactivity	B	Negative
B	Anti A with known reactivity	A	Negative
A	Anti A with known reactivity	A	Positive

Analogous control reactions were made for anti B test sera

Tables 1 and 2 A serum of known reactivity was included in all tests as a positive control

RESULTS

As the epithelial cells tested in the MCA reaction were from the spinous layer, the results of the immunofluorescence reactions were read in this layer so that comparison between the two reactions was possible

When the immunofluorescence staining method was positive, green fluorescence was seen on all the cell membranes of the epithelial cells except the basal cells The positive reactions were identical in sections from group A and group B subjects

Haematoxylin eosin staining of the sections showed normal nonkeratinized stratified squamous epithelium

In the MCA reaction, weak results (as judged by the reduced number of cells which showed agglutination) were generally accompanied by a diminished number of red cells adherent to the individual tissue cells

In the strong MCA reactions it was noted that there were always some cells which did not react

Anti A Sera

IF staining Fourteen out of 18 tested sera produced positive IF staining In 9 of the 14 positive cases the titre for the anti A serum was higher when SwAH IgM/FITC® was used, in only three cases it was higher when SwAH IgG/FITC® was used, Fig 1

Further, if SwAH IgG/FITC had been used as the only conjugate, no more than 8 (instead of now 14) sera would have produced positive staining Fig 1

MCA reaction and comparison between the IF and MCA reaction Only 9 blood group antisera were able to produce the MCA reaction

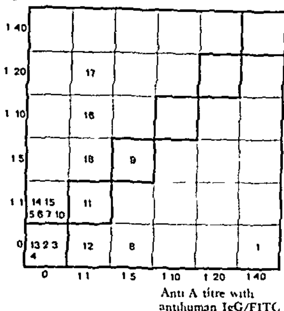


Fig 1 Comparison between anti A titres when anti human IgM/FITC and antihuman IgG/FITC are used as second layer Each number represents an anti A serum, and the same number is used for the same anti A serum in all the figures

None of the sera which failed to react in the IF staining (sera No 2, 3, 4, 13) were able to produce positive mixed cell agglutination

When corresponding titres in the IF staining and the MCA reaction were compared using the highest of the two IF titres in every case, it was seen that the highest titre was obtained in the IF staining in 8 cases and in the MCA reaction in two cases, Fig 2

Anti B Sera

IF staining All tested anti B sera reacted in the IF staining method

In 7 cases the titre was higher when SwAH IgM/FITC® was used and in three cases it was higher when SwAH IgG/FITC® was used If SwAH IgG/FITC® only had been used, 12 (instead of now 16) sera would have reacted positively, Fig 3

MCA reaction and comparison between the IF and MCA reaction Eleven blood group antisera were able to produce the MCA reaction When comparison was made between corresponding titres of blood group antisera able to react in both the IF staining method and the MCA reaction, only two sera had a titre in the MCA reaction which was higher than the highest of the two titres in the IF staining method, in 8 cases the titre obtained in the IF staining method was higher than the titre in the MCA reaction, Fig 4

Control reactions Except for the positive controls included all control reactions were negative

The non reactivity of labelled anti IgG globulin in reactions where labelled anti IgM globulin was positive, and vice versa, substituted for controls of labelled normal serum against a complex of antigen and specific antiserum

DISCUSSION

The present study has shown that careful selection of blood group antisera is necessary

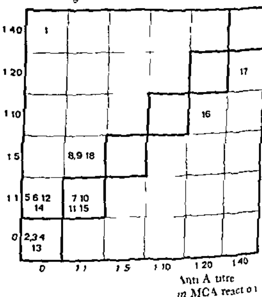


Fig 2 Comparison between the anti A titres in the IF and the MCA reactions Each number represents an anti A serum and the same number is used for the same anti A serum in all the figures

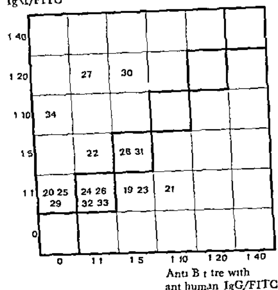


Fig 3 Comparison between anti B titres when anti human IgM/FITC and anti human IgG/FITC are used as second layer Each number represents an anti B serum and the same number is used for the same anti B serum in all the figures

in developing a sensitive technique for the demonstration of blood group substances A and B in tissue sections

Also it is more likely to find sera that will react in the IF technique than in the MCA reaction however in the two different techniques the titres of the reactive sera are of approximately the same magnitude

Part of the explanation why more sera react in the IF staining method than in the MCA reaction is that although the IgG fraction of the blood group antisera will not give a positive MCA reaction as often as the IgM fraction (1 7) it does react with the tissue cells (7) and will thus produce positive IF staining

The ability of the sera to react with blood group substances A and B may be due to either IgM or IgG or both This stresses the need for using conjugated antisera to both immunoglobulin classes in order to select the most sensitive system and it is clearly demonstrated by the fact that as many as 10 out of 31 positive stainings would have failed to react if only antihuman IgG conjugate had been used It is important to notice that most unfractionated, commercially available, anti human globulins chiefly consist of anti IgG with only low concentrations of anti IgM

The reason why only some of the anti A sera can be used in the IF technique cannot be fully explained by the variation in amounts of specific IgM and IgG in these test sera This is illustrated by the peculiar finding that four anti A sera with adequate haemagglutination properties could not be used to demonstrate blood group substances in tissue cells with the IF method Further characterization of factors of importance for the reactivity of anti A and anti B sera in the IF staining method is of great interest but has not been investigated in the present study

In the IF staining method the positive reaction is seen in all cells except the basal cells This is in contrast to the MCA reaction

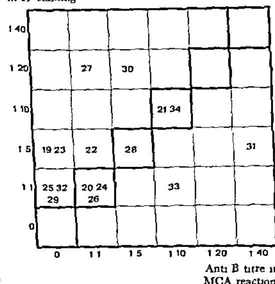


Fig 4 Comparison between the anti B titres in the IF and the MCA reactions Each number represents an anti B serum and the same number is used for the same anti B serum in all the figures

where some non reacting spinous cells always are in evidence The results of the present investigation fail to explain this phenomenon but in this respect it seems that the IF staining method is more sensitive than the MCA reaction

The filter system (14 15) used for fluorescence microscopy is of importance for the high sensitivity of the technique The main advantages of the interference filter are the bright specific immunofluorescence and the absence of autofluorescence which facilitate reading of the slides

The development of a sensitive method for demonstrating blood group substances in formalin fixed paraffin embedded tissue is of great interest as a partial or complete loss of these blood group substances has been reported in premalignant and malignant diseases arising from epithelium in which such substances are normally present (2 3 4 9 10 13)

It has been shown (10) that blood group substances due to their polysaccharide nature are not influenced by the formalin fixation and paraffin embedding procedure As this fixation and embedding procedure has for years been the methods of choice for most routine histopathological work it opens up the possibility to investigate the presence of blood group substances in large surgical materials and further to combine the IF method with routine histological examination

In conclusion the study has shown that by selection of anti A and anti B sera according to titres obtained in a serial dilution IF staining reaction by matching antiserum and conjugate and by optimizing the optical system it is possible to produce a highly sensitive immunofluorescence method for the demonstration and localization of blood group substances A and B in oral epithelium This method seems to be as sensitive as the MCA reaction but is superior to the latter in allowing a more accurate localization of the antigens

The present work was supported by Grant DE 1358 from *The National Institute of Dental Research National Institutes of Health Bethesda Maryland USA*, *Vera og Carl Johan Michaelsens legat* and by Grant from the *Danish Medical Research Council*

The authors want to thank Dr *J Andersen* head of Blood Grouping Department Statens Serum institut Copenhagen and Dr *H Sorensen* Department of Serology University Institute of Forensic Medicine Copenhagen for kindly providing antisera

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Acta path microbiol scand Section A, 80 440–448, 1972

AN ELECTRON MICROSCOPICAL STUDY OF NUTRITIONAL MUSCULAR DEGENERATION (NMD) OF MYOCARDIUM AND SKELETAL MUSCLE IN CALVES

AILI OKSANEN and RITVA POUKKA

Department of Pathology and Department of Biochemistry, College of Veterinary Medicine Helsinki, Finland

NMD provoked by diets containing profuse quantities of unsaturated fatty acids occurred in three calves, while four other calves were protected with vitamin E The diseased calves presented macroscopic and light microscopic waxy degenerative changes typical of NMD Electron microscopy of degenerated areas in the myocardium and in skeletal muscle, revealed that the presence of strongly electron-dense formations between intact myofibrils represented the first stage in the disease The localization size and shape of these formations were consistent with features in the mitochondria of intact cells In the same areas intact mitochondria were mostly absent In some areas, mitochondria with only some electron-dense crystals could be seen In further advanced stages of the disease, similar electron-dense formations might be seen but they were surrounded by a structureless, necrotic mass without myogenic striation Abundant, immature cells, most of which were difficult to identify were seen between the destroyed muscle cells The cells which had the nature of satellite cells and were located below the intact external lamina of the degenerated cell were intrepreted as myoblasts

Nutritional muscular degeneration (NMD) is a selenium- and vitamin E responsive disease to occur especially in calves, lambs and pigs, it occurs locally in various parts of the world In NMD, waxy degeneration involving the myocardium and, symmetrically, skeletal muscle is observed numerous histological descriptions have appeared in recent years (for references, see *Oksanen* 1965) NMD can be provoked by excessive unsaturated fatty acids if the animals are not simultaneously supplied with anti oxidants in adequate amounts as protection (*Adams et al* 1954 *Blaxter & McGill* 1955)

Although the histological picture of NMD is highly typical and well known, the involved changes observed by electron microscopy and described in the literature have been concerned only with advanced degenerations of the skeletal muscle (*van Vleet et al* 1968) The initial phase of the disease is still obscure An electron microscopical study of the myocardium and skeletal muscle of calves with NMD was therefore considered appropriate

MATERIAL AND METHODS

Seven calves of Ayrshire breed were used in this experiment They came from different frams where they were fed with colostrum and whole milk during the first week of life On the eighth day, the calves were divided into two groups One group of four calves received vitamin E which was withheld from the three calves in the other group In both

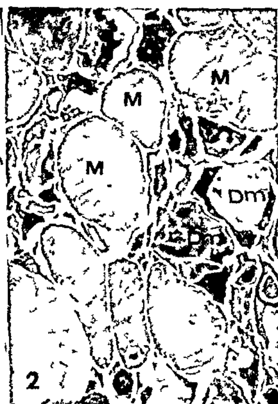
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g oups m lk fat was replaced by v tam n E free corn o l (Eastman S ripped Corn O l Eastman Kodak Company Rochester N Y U S A) ob ta ned by homogenizing skim m lk and o l in a Warring Blendor for about 5 m nutes prior to feed ng The cal es were g ven 4 l of f lled m lk per day throughout 17 days To this rat on 400 mg of dl α tocopheryl acetate (F Hoffman La Roche Co Ltd Basle Sw tze land) were added and g en to animals n the v amin E group In both groups 5800 I U of trans Ret nol Acetate (S gma Chemical Company) were added Anti b ot cs aga nst diarrhoea vere adm n s ered to all calves

The cl n cal appearance of the cal es and the r SGOT (serum glutam c-oxaloacet c transam nase) values were followed When muscular degenerat on was observed the experimental an mal conce ned and one control were s multaneously sacrif ced at the age of 21 25 days Immed ately af er slaughter ing samples were taken for h stolog cal and elec tron m croscop cal examinat ons as well as for l p d analys s the results of wh ch are to be separately reported (*Poukka & Oksanen* 1972) The samples from the f rst slaugl tered calf we e taken from the degenerated a eas of M serratus ventralis M vastus intermed u M splen us furthermore from a macroscop cally intact s te in the M vastus inter med us and from the myocard um subendocardial ly as well as from the centre of the septum Samples from the same s tes v ere obta ned from all the other calves

W h a view to examinat on by *l ght m roscope* frozen sect ons of 10 µm th ckness fixed n formal n were sta ned w h scarlet red and Sudan black From the prepara ons embedded in paraffin sec tions of 5 µm were made and sta ned w h haema toxyl n eos n w th Kós a s calc um sta n w th





F g 1 L ght m crograph showing vaxy degeneration. The area conta ns cells with intact muscle f brils (M) alternating w h strongly degenerated *f bres (Dm)* of fuch only swollen fragments might be is ble New young cell nucle surrounded them Glutaraldehyde and osmium tetrox de fixation Tolu d ne blue sta n ng × 1 300

F g 2 L gl t m crograph of *trans erse sect* on of skeletal muscle w th NMD Intact (M) and degen era ed f bres (Dm) al ernate R ch cell prol ferat ons Glutaraldehyde and osm um tetroxide f xation Tolu d ne blue sta ning × 1,300

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Histological examination revealed typical waxy degeneration of various forms, degrees and intensity in all experimental animals Intact muscle fibres alternated with degenerated ones and nuclear proliferation was seen near the sarcolemma of muscle cells or *in extensive areas between intact fibres* (Figs 1 and 2) The cytoplasm of larger proliferated cells acquired yellow stain with van Gieson The changes in the myocardium were slight In two of the control animals very few degenerated fibres were seen in sections of skeletal muscle No fat was noted in the muscles or myocardium of the controls, but in the experimental animals muscle fibres free of waxy degeneration might be sites of slight fatty degeneration in the form of fine droplets The calcium and iron stains produced negative results in the muscles and myocardium of all calves

Electron microscopy of *intact skeletal muscle* disclosed the typical picture of muscle, with distinctly recognizable sarcomeric striation in the myofibrils Mitochondria a sarcoplastic reticulum forming light coloured gaps and glycogen in the form of small osmiophilic granules between the myofibrils were observed The nuclei were oval and located on the margins of the cells In the *intact myocardium*, the nuclei were rather irregular in shape and located in the centre of the cell The mitochondria were seen in the interstices of the contractive material (Figs 3–5) most abundantly in the vicinity of the nucleus The sarcomeric striation was distinct (Fig 4) Desmosomes could sometimes be seen between plasma membranes of two myocardial cells In longitudinal junctions intercalated discs were observed (Fig 5)

In the *degenerated areas* intact and altered cells alternated (Figs 3–5) In the cells in which changes were slightest intact myofibrils with distinct sarcomeric striation were seen (Fig 4) Electron dense formations were seen to be interspersed in the contractile material (Figs 4–5) These formations were resolved into distinct groups of crystalline like electron dense formations (Fig 5) The size shape and localization of these electron dense formations were similar to those of the mitochondria of intact cells In muscle cells containing these electron dense formations intact mitochondria were uncommon (Fig 3) In some areas however, mitochondria with partly visible cristae and electron-dense crystals could be seen Areas of more advanced changes also comprised the same electron-dense formations but the striation of muscle cells had disappeared and was replaced by a structureless necrotic mass (Fig 7)

It applies to the skeletal muscle as well as to the myocardium that a cluster of proliferated cells was seen outside the muscle cells They had a cytoplasm presenting mitochondria intermembraneous vacuoles and vesicles and a rich endoplasmic reticulum with numerous ribosomes (Figs 7–9) In degenerated skeletal muscles below their intact external lamina satellite cells in various developmental stages were seen surrounded by their own sarcoplasm Some of these cells had several nuclei (Fig 7)

DISCUSSION

The present material displayed nutritional muscular degeneration provoked by abundant unsaturated fatty acids All the animals not protected with vitamin E were affected The macroscopic and microscopic pictures conformed to those repeatedly described in the literature on spontaneous as well as provoked

Fig 6 Electron micrograph of the myocardium of a calf with NMD Two mitochondria The right one crowded by electron dense crystals (Db) The left one shows intact membrane and some intact christae (C) and electron dense crystals (Dc) in some areas × 85 000

Fig 7 Three degenerated skeletal muscle cells (Dm) with electron dense bodies and necrotic myogenic substance Within the same external laminae (El) satellite muscle cells (Sc) encircled by their own sarcoplastic membrane In their cytoplasm intact mitochondria and tubular organelles Nucleus of the satellite cell (N) The satellite cell at the bottom has three nuclei In the centre a capillary and a proliferated cell × 9 800


Ob
C
6
N
Dm
Dm
Sc
El
Se
7
N
Sc
El

NMD (*Nieberle* 1926, *Slagsvold & Lund Larsen* 1934, *Hjärre & Lilleengen* 1936 *Swahn et al* 1948, *Marr et al* 1956)

Electron microscopical study of intact myocardial and skeletal muscle revealed normal areas with sarcomeric striation, sarcoplastic reticulum, and mitochondria In the myocardium, desmosomes and intercalated discs (*Caulfield & Klionsky* 1959, *Meessen* 1967) resembling the corresponding structures in normal tissues were seen (*Sommer & Johnson* 1969, *Fawcett & McNutt* 1969, *Martinez Palomo et al* 1970)

In light and electron microscopy, both intact and affected muscle cells were seen in the degenerated areas The first and most striking changes were the electron dense granular formations of a size, shape and localization in the cell corresponding to features of mitochondria of intact cells Occasionally mitochondria with some intact cristae and some electron dense crystals representing the developmental phase of these formations could be seen It seems likely that the said formations represent changed mitochondria This hypothesis is also supported by phospholipid analyses of the same muscle samples in which increased percentage of non mitochondral sphingo myelin (*Fleischer & Rouser* 1965) and partial descrease of cardiolipids (*Poukka & Oksanen* 1972) were noted

It appears likely that the mitochondria of cells are first damaged in metabolic disturbance caused by feeding with excessive quantities of unsaturated fatty acids in the absence of an anti oxidant This is further supported by the fact that more than half of the muscular GOT is located in the mitochondria (*Körmendy et al* 1965) and that the first clinical symptom of NMD is the elavated SGOT (*Oksanen* 1965)

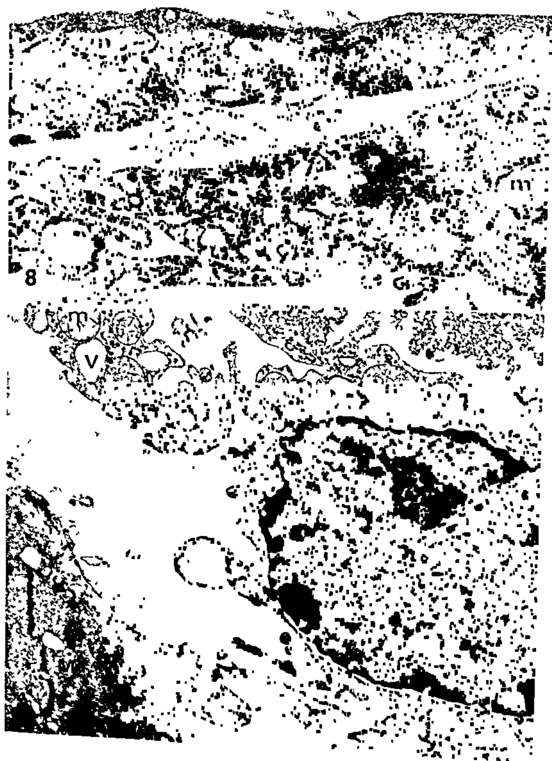
As regards the chemical composition of this electron dense material nothing definite can be said on the basis of morphology In muscular degeneration calcifications have often been seen histologically (*Hjarre & Lilleengen* 1936 *Swahn et al* 1948 *Marr et al* 1956 *Andersson* 1960) Calcium however was not seen in the light microscopy of the same areas Triglycerides are osmiophilic, but the fat droplets appear in the electron micrograph as structureless black spots (*Wolbert* 1968) Greater abundance of triglycerides is also unlikely considering the negative results obtained by light microscopy after scarlet red staining of the degenerated areas. In older animals, lipofuscin is frequently seen as a brown atrophy in the mitochondrial area of the myocardium but its structure differs from that seen in the present study (*Fawcett & McNutt* 1969) However these facts do not exclude a possible presence of other lipids such as free fatty acids

In the present material, abundant immature cells appeared both in the myocardium and in skeletal muscle These were seen in connection with the external lamina or they were situated between the myofibres Especially the latter were difficult to identify positively Their well developed endoplastic reticulum rich in ribosomes indicate a lively proteinsynthesis Their morphology bears a strong resemblance to the pericapillary histiocytes which rapidly increase in connection with myocardial ischaemia (*Hasper* 1964 *Buchner & Onishi* 1968) On the other hand according to *Allbrok* (1962) the proliferated cells are macrophages as well as fibroblasts and myoblasts which are difficult to differentiate

The immature cells seen under the external lamina of the degenerated skeletal muscle cell but encircled and separated from the old cell by their own sarcoplasm resemble the satellite cells first described by *Mauro* (1961) in connection with muscular regeneration and later described also by *Allbrook* 1962 *Shafik & Gorycki* 1965 *Reznik* 1969

Fig 8 Proliferated cell between skeletal muscle fibres Mitochondria (m) and rich endoplastic reticulum (Er) with ribosomes in the cytoplasm × 40 000

Fig 9 At the bottom skeletal muscle cell (M) In the centre a proliferated cell with mitochondria (m) vesicles (V) and endoplastic reticulum with ribosomes in the cytoplasm × 20 000


8
m
G
V

The electron microscope pictures are taken in the Electron Microscope Laboratory, University of Helsinki

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ELECTRONMICROSCOPICAL STUDY OF MUSCLE BIOPSIES FROM HEALTHY YOUNG PEOPLE

Methodology and Results

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Electronmicroscopy has been performed on biopsy material from human striated muscle—obtained on the basis of voluntariness and good-will of eleven young, healthy students The results are presented in this paper, which deals with the following two main subjects 1 Information concerning the technical procedures used in this laboratory with remarks on certain important points at different stages in the preparation 2 The results of an extensive electronmicroscopical investigation of these biopsies which revealed a rather pronounced morphological variation in young healthy, human subjects The classic electron microscopic morphology of striated muscle has in reality for many years been based on animal experiments This investigation in healthy human subjects has demonstrated a morphology basically identical to that of animal muscle, but it seems as if additional elements must be regarded as necessary parts of a true electronmicroscopic picture of striated muscle in man

Morphological investigations of striated muscle from diseased human beings are often a valuable diagnostic aid The classic staining methods however, may only provide information of limited value in the actual diagnostic considerations Additional special procedures such as intravital staining of the intramuscular nervous system, histochemistry and in the last decades even electronmicroscopical studies may

1 Widen our knowledge of already established histopathological entities
2 Help in the dissociation of diseases and syndromes
3 Reveal quite unknown evidence of new diseases

In the treatment of biopsy material the electronmicroscopical technique is complicated and time-consuming and demands special care at all stages in the procedure, but this tool is an important and fascinating supplement to the conventional examination of biopsies In the current discussion concerning morphology, function and biochemistry of striated muscle the electronmicroscopical evidence has often been a stimulating—perhaps even deciding—element

Received 23 xii 71

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TABLE 1 *Essential Personal Data*

No	Sex	Age (year)	Length (cm)	Weight (kg)	Muscle	Sport
111/6662	♀	28	158	53	*Musculus peronæus brevis sin*	
225/10 171	♀	27	166	64	Musculus tibialis anterior sin	Riding
233/10 420	♂	29	188	90	Musculus tibialis anterior sin	Running
235/10 437	♂	22	175	61	Musculus tibialis anterior sin	
236/10 473	♂	22	170	62	Musculus tibialis anterior sin	
237/10 198	♂	23	182	72	Musculus tibialis anterior sin	
238/10 551	♀	24	178	68	Musculus palmaris longus sin	Figure skat
242/10 589	♀	19	168	54	*Musculus palmaris longus dxt*	Athletics
244/10 629	♂	20	180	69	Musculus palmaris longus sin	Football
251/10 780	♀	21	158	56	Musculus tibialis anterior sin	
252/10 804	♀	20	169	63	Musculus palmaris longus sin	

A leading problem is the constant need of a control material obtained from healthy subjects In the neuropathological department, Aarhus Municipal Hospital, we have now on the basis of good will and voluntariness of 11 students received biopsy material from striated muscle which has been examined in the light microscope as well as in the electronmicroscope

MATERIAL AND METHODS

The material (Table 1) includes 11 students—6 women and 5 men 19 to 29 years of age The height of the women was 158 170 cm of the men 170 188 The weight of the men was 62 90 kg of the women 53 68 kg *All students had essentially* been healthy earlier one was lefthanded and one had had a normal delivery 6 years before the biopsy Five students went in for sports while the rest did not participate in such activities or other kinds of hard work The biopsies were taken from the long palmar muscle (3 ♀ and 1 ♂) from the *anterior tibial muscle (2 ♀ and 4 ♂), or from the* short peroneal muscle (1 ♀)

Methods*

As described elsewhere (*Reske Nielsen et al* 1969) the specimen for electronmicroscopy was fixed in glutaraldehyde and osmium tetraoxide dehydrated and embedded in Vestopal W in gelatin capsules. After hardening the material is ready to be cut In the preparation of the biopsy material for electronmicroscopy the following apparature has been *employed*

Zeiss Light and Stereomicroscope **
LKB 4800 A Ultrotome ***
LKB 7800 A Knifemaker ****

The preparation procedures have been performed as described by *Pease* (1964) However a few remarks on the technical details are necessary as they are not mentioned in available brochures or in the literature concerning ultra thin sectioning

The sectioning was done with glass knives Preliminary thick sections (about *2000 Å*) *were collected* and transported to a slide by a simple but *important instrument which* is made in the following way A metal eye (2 mm in diameter) is formed at one end of a wire (diameter about 0 5 mm) the other end of which is inserted into a heated glass rod The preliminary thick sections *are caught by the metal eye*

The thin sections (500–600 Å) are first collected in the basin at the glass wedge *with a suitably* stiff vibrissa which is fixed in a rod with ordinary tape caught by the grid and after drying stained by double staining with Uranyl magnesium acetate and lead citrate (*Reynolds* 1963 *Pease* 1964 and *Frasca & Parks* 1965)

To avoid precipitation of lead carbonate (contamination with atmospheric carbon dioxide) in the sections the grids are placed in a Petri dish which has been transformed into a *carbon dioxide free* chamber by moistening the filter paper at the bot

* *A duplicate with essential phototechnical data and a detailed description of every single step in the preparation procedure for electronmicroscopy including the staining methods used especially for muscular tissue in this laboratory is available on request together with reprints*

** Consult instructions for Zeiss Microscopes
*** Consult instructions for *LKB 4800* A Ultrotome
**** Consult instruction for LKB 7800 A Knifemaker

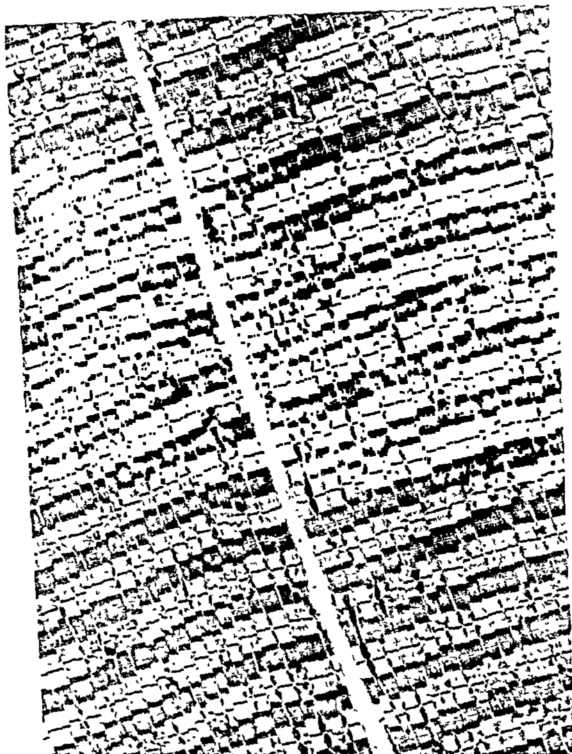


Fig 1 Striated muscle in longitudinal section For legend See text. (5000 ×)

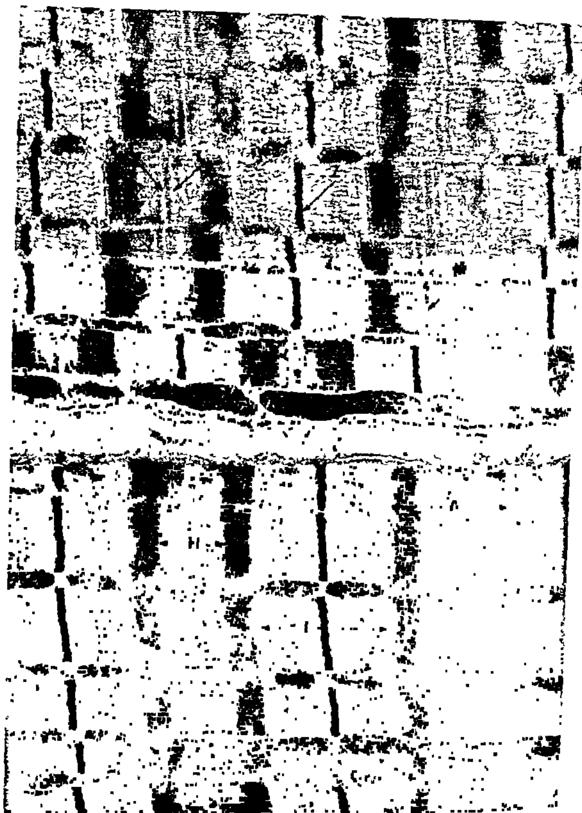


Fig 2 Striated muscle in longitudinal section For legend See text (18000 ×)

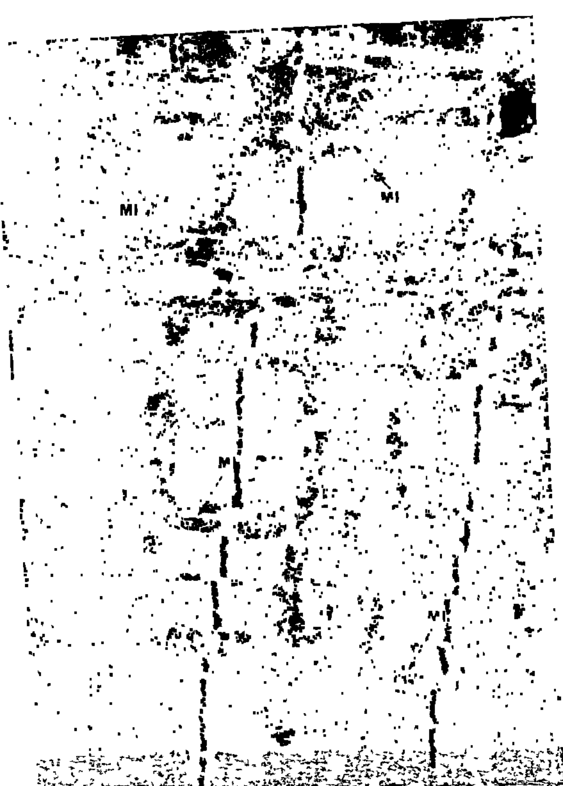


Fig 3 Striated muscle in longitudinal section At the top a fibre rich in mitochondria is seen—at the bottom a fibre poor in mitochondria (18000 ×)



Fig 4 Striated muscle in longitudinal section MI A large bag of mitochondria is seen in the central part of the picture LI Lipid bodies (18000 ×



Fig 5 Striated muscle in longitudinal section L A lipid droplet is located between two fibrils (27000 ×)

tom with lead acetate upon which the lid is put on It is even important not to breathe onto the sections when the lid is off After staining, rinsing and drying the sections are ready for examination in the electron microscope (Zeiss EM 9 S)

For the photographic procedure an Agfa Planfilm Scientia 23 D 5 (7 × 7 cm, Orthochromatic, 10 din) is used and, in the daily routine, it is recommendable to use a quick development system (Rapidoprint a o) Photo material for reproduction, clichées and dias should always be done by specialists

ELECTRONMICROSCOPICAL RESULTS

Five blocks with 6 grids from each have been examined per student, i e a total of 30 grids from each biopsy

Classical Picture

In our human material we have found all the elements which are regarded to be parts of the generally accepted, classic picture of striated muscle Every fibre is composed of fibrils and these again of filaments The characteristic, longitudinal striation is produced as a consequence of the special morphology The *A substance*, composed of thick filaments (150–180 Å in diameter) and the *I-substance*, composed of thin filaments (approx 50 Å in diameter) bring about the longitudinal striation The cross striation is brought about by other structures The *Z-membrane*, a zig-zag structure which binds the single filaments together and at the same time delimits the sarcomeres The *H-membrane*—the area where the thin filaments of the I substance do not interdigitate with the thick filaments of the A substance The *M-line*—located in the middle of the A substance and having an extra density caused by the so called connecting rods which combine and fasten the A-filaments The *L-line*—

being located to both sides of the M-line and being lighter which is caused by the lack of the so-called bridges from the A-filaments. (Figs 1, 2). The mitochondria are seen between the fibrils Some fibres are rich in mitochondria (Type B and C = red fibres = type 1) which are large, numerous and branched Others are poor in mitochondria (Type A = white fibres = type 2) which are small, lying as twins at the Z-membrane in a characteristic way. (Fig 3). Sometimes these organelles lie in large "bags" beneath the sarcoplasma membrane, (Fig. 4). A part of the classic picture are small drops of fat between the fibres. (Fig 5) The sarcotubular system is constructed by the transverse tubular system (T-system) and the sarcoplasmatic reticulum (SR) and is located between the fibrils (Fig 6). Every fibre is delimited by a membrane—the sarcolemma—stratified with 3 layers (about 75 Å) under which nuclei with nucleoli are localized The space between two fibres contains collagen fibres and capillaries. Between the fibrils and the sarcotubular system, glycogen granules are found (Fig 7) The neurogenic end plate is an important structure of every muscle fibre and forms synapses with the subneural apparatuses which are specialized parts of the sarcoplasma membrane (Fig 8).

Additional Observations

This electronmicroscopical investigation of biopsy material from 11 healthy students has revealed the following elements which are not parts of the generally accepted classic picture of striated muscle

The fibril calibre may vary much (Fig 1-4) and this may be quite a dominating feature In correctly cut longitudinal sections the

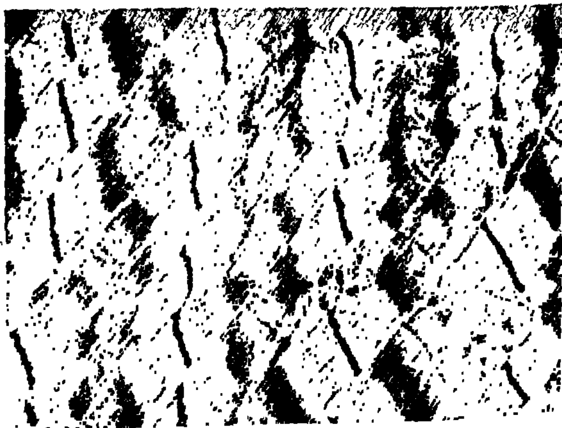


Fig 6 Striated muscle in obliquely cut section, which displays the tubular systems we[illegible] system SR Sarcoplasmatic reticulum (18000 ×)



Fig 7 Striated muscle in longitudinal section S Sarcolemma membrane CO Collagen NU. Nucleus GLY Glycogen granules (18000 ×)



Fig 8 Motor end plate in striated muscle AX Axon S Sarcolemma membrane SV Synaptic vesicles SC NU Schwann nucleus (18000 ×)

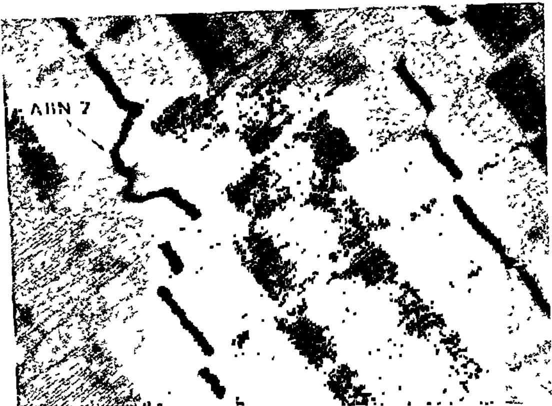


Fig 9 Striated muscle in longitudinal section ABN Z Note the characteristic changes in the configuration of the Z membrane (27000 ×)

Z membranes are often displaced in parallel so that the linear continuity is broken (Fig 1)

Another peculiarity is a *characteristic change in the configuration of the Z membrane* (Fig 9)

In other sections these changes are more marked, reaching degrees of complete disorganization (*flowing Z membranes or streaming Z discs*) (Fig 10)

In all volunteers, a varying number of *osmophilic bodies*" can be found between the fibres—often close to the mitochondria They present a rounded shadow with small vacoules which are not electron dense (Fig 4) Similar structures with zebra like striation may be seen (*"zebra bodies"*) (not illustrated)

Sometimes finely laminated bodies are seen within the mitochondria (*"myelin bodies"*) (not illustrated)

In a few preparations, vaguely demarcated areas between the fibrils—and considerably broader than these—are seen to be built up by *membranes of varying electron density* (Fig 11)

Under the sarcoplasma membrane, *tubular structures* can be seen to cross each other at different angles—peripherally delimited by a fillet of mitochondria (Fig 12)

The picture may vary from student to student—especially as regards the amount of osmophilic bodies which seem to be more numerous in the anterior tibial muscle than in the long palmar muscle A female student (111/6662) displays quantitative alterations compared with the other students Her osmophilic bodies are much more numerous, her fibrils are much more varying in diameter, and the changes in her Z membranes are more pronounced Even in the light microscope her biopsy differs from those from other



Fig 10 Striated muscle in longitudinal section STR Z Streaming Z-discs (18000 ×)



Fig • longitudinal section ME Note the numerous membranes of varying electron unidentified nature (18000 ×)

volunteers who present the commonly described normal picture (*Coërs & Woolf* 1959, *Adams et al* 1962, *Walton* 1964, *Reske-Nielsen et al* 1970 *Evans* 1970)

She has a few lipocytes between the fibrils, swellings along the axons of the intramuscular nervous system and sprouting This student was 28 years old and the biopsy was taken from the short peroneal muscle Before she started studying medicine she was a nurse —a job which extensively strains the lower extremities She had never been seriously ill

Whether the age of this student, the choice of muscle or perhaps her earlier occupation may have been responsible for these findings or whether they should be regarded as normal variations in healthy persons is not possible to decide



Fig 12 Striated muscle in longitudinal section TU Tubular structures are seen to cross each other at different angles LY Lysosome CO Collagen S Sarcoplasmatic membrane (18000 ×)

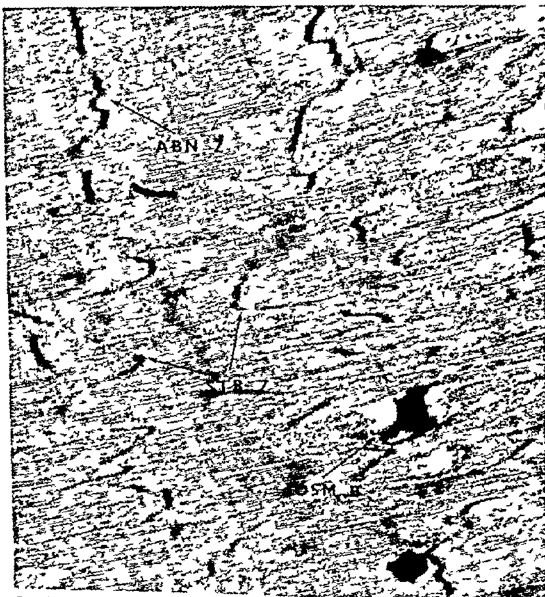


Fig 13 Recently d agnosed d abetes mellitus S ria ed muscle in longitud nal sect on ABN Z Abnormal Z-membranes STR Z Stream ng Z-d cs OSM B O moph l c bodies Note the characteristic kn fe marks (18000 ×)

DISCUSSION

The electronmicroscop cal investigat on of riated musculature from young healthy human be ngs 19 29 years old confirms the ge rally accepted electronm croscopical pic re s de cribed in works about neuro m lar diseases *Adam s et al* 1962 *Rl odin* 1 1 *Walton* 1964 *Fawcett* 1966 *Pellegrino* & *Fran ini Armstrong* 1969 *Walton et al* 1969) This class c morphological description has been built up on the bas s of electron microscopic l exam nat ons of a large number of an mal species *

* For references see J Ul ras ruct Res J Cell B ol Exp Mol Path

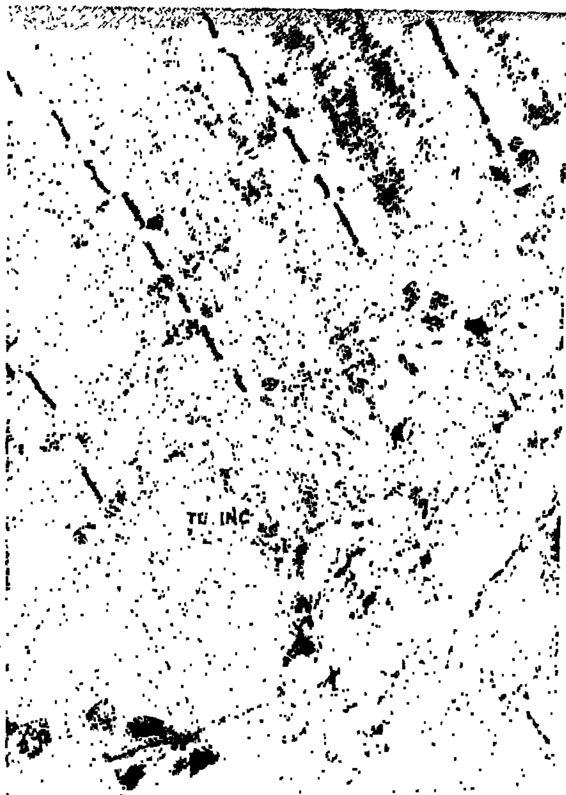


Fig 14 Striated muscle in longitudinal section TU INC Tubular *inclusion* of unidentified nature MI Mitochondria (18000 ×)

Through a close collaboration between many research workers in different scientific fields, i e biochemistry, physiology a o, it has been possible to correlate morphology and function and thereby the mechanism of contraction in striated muscle (*Huxley* 1953, *Huxley* & *Niedergerke* 1954, *Huxley* & *Hanson* 1954, *Huxley* 1956 *Spiro* 1956, *Sjostrand* & *Andersson* 1956, Carlson *et al* 1961)

The morphology of the transverse tubular system and the sarcoplasmatic reticulum—the so called T SR system—has been described earlier and its function in relaxation excitation has been proven (*Huxley* 1960, *Hasselbach* & *Elfvin* 1967, *Pellegrino* & *Franzini Armstrong* 1969, *Kelly* 1969)

The examination of striated muscle in healthy, young people reveals the same structures as in animals, and the physiological and biochemical processes are presumably linked up with the analogous structures, as well as the triggering mechanisms probably are identical

Few electronmicroscopical investigations of striated muscle from human beings are available *de Harven* & *Coërs* described in 1959 the first human end plates which were found in tissue samples from diseased persons *Van Breemen* (1960) found among 55 patients 10 biopsies which were described as normal *Luft et al* (1962) has examined striated musculature from a healthy person and compared it with several muscle biopsies from a patient with non thyroid hypermetabolism The patient and the control were directly comparable as regards age sex and choice of specimen

Systematic electronmicroscopical investigations in healthy young people have not been available up till now

Even though some workers have succeeded in showing that some diseases imply primary defects in the mitochondria (*Shy et al* 1963, *Shy* 1966, *Shy* & *Magee* 1966, *Shy et al* 1966 *Price et al* 1967, *Sluga* & *Moser* 1970, *Buscaino* 1970) and that some myopathies imply changes in the T SR system (*Biczyskowa et al* 1969) the findings being based on electronmicroscopical investigations in striated muscle, the electronmicroscopical findings in neuromuscular diseases are often of unspecific nature (*Palmeiro et al* 1966, *Schotland* 1969, *Engel* & *MacDonald* 1970, *Tomonaga* & *Sluga* 1970, *Fardeau* 1970, *Mair* 1970)

Our findings in some patients with neuromuscular diseases, e g muscle biopsies from patients with recently diagnosed and long term diabetes mellitus have revealed similar changes—only qualitatively and quantitatively more pronounced—as we have found in healthy persons variations in fibril diameter, changes in the configuration of the Z membranes and osmophilic bodies (Fig 13) Even inclusions of the same character are seen (Fig 14) We therefore have the impression that the borderline between normal and pathological conditions is by no means distinct

On the contrary, a most cautious interpretation of the electronmicroscopical findings is necessary if important pathographic mistakes are to be avoided

We have concluded the following on the basis of this electronmicroscopical investigation in muscle biopsies from 11 young, healthy volunteers

1 At certain points it is necessary to add new elements to the classic, electronmicroscopical description of striated muscle in man, which in reality for many years has been based on animal experiments A varying fibril diameter, a characteristically changed morphology of the Z membranes, "osmophilic bodies in varied numbers and appearance, and structures—sometimes of tubular appearance—the nature of which is not explicable at present
2 It seems necessary to stress the morphological variations in young, healthy people
3 As long as our knowledge in this respect does not permit us exactly to delimit normal variations from pathological changes, it is absolutely necessary to provide a sufficient control material which must be comparable as regards age, choice of muscle, training condition e t c

4 Examination of specimens requires a meticulous observation technique

5 A skilful and experienced technical group and a close collaboration with specialists from other fields (Neurosurgery, neurology a o) are conditions *sine quibus non* in the evaluation of the electronmicroscopical findings

6 The preparation of striated muscle for electronmicroscopy is a delicate procedure applied to an extremely sensitive tissue A comparison of morphological results from one laboratory with those from another laboratory implies a detailed knowledge of the procedure used for the preparation

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Acta path microbiol scand Section A, *80*, 468–470, 1972

THE EFFECT OF SYNGENEIC TRANSFER OF NORMAL LYMPHOID CELLS ON THE DEVELOPMENT OF CASEIN-INDUCED AMYLOIDOSIS IN MICE

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Mouse amyloid induction with casein was accelerated by weekly injections of 10^9 syngeneic non sensitized lymph node cells Injections of spleen cells and of a mixture of thymus and bone marrow cells were without effect A slight drop in the level of circulating casein antibodies was noticed in mice having received lymph node cells Two explanations of the acceleration were considered An accelerating factor may be released from desintegrating lymph node cells or lymph node cells are producing the amyloid fibrils which later lodge in the spleen

Induction of amyloidosis has been postulated to depend on dysfunction of the cellular immune apparatus, induced by a prolonged challenge with antigen (*Teilum* 1964, *Ranløv* 1968, *Hardt* 1971b) We have therefore examined the influence of the transfer of normal lymphoid cells to syngeneic mice undergoing casein-induced amyloidosis as this transfer might prevent the break-down of the immune apparatus

MATERIAL AND METHODS

The animals were randomly selected inbred C3H mice, weighing from 23 to 27 Gm at the beginning of the experiment They were fed on oats

Donor cell suspensions were prepared from 8 weeks old untreated C3H mice Spleens lymph nodes and thymi were homogenized in a Potter Elvehjem and washed three times in Hank's Stock Solution (HSS) Bone marrow cells were obtained by flushing the bone marrow with HSS Furthermore, homogenate of spleen cells were prepared by sonicating the cell suspension for 15 sec with a Bronson Sonifier 20 KH After this treatment no intact cells could be found in trypan blue stained suspensions

Recipients were treated with daily s c injections of ½ ml of a 5 per cent solution of sodium caseinate subcutaneously for 4 weeks (5 times a week) On days 7, 14 and 21 the mice received lymphoid cells or homogenate hereof i v as stated in Table 1 All animals were killed at day 28 The spleen, liver and kidney were taken for microscopy and stained with haematoxylin eosin methyl ... d the P A S. ..., and ...ossed

polars

The degrees of spleen amyloidosis were graded according to the semiquantitative method of *Christensen & Hjort* (1959) a thin continous ring of amyloid around a spleen folicle being rated as grade 3

Circulating antibodies to casein Determination

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TABLE 1 *Experimental Design and Results*

Transfer of	No of recipients	Amyloidosis incidence	Average of spleen amyloidosis	Casein antibodies Radial diffusion diameter in mm
Spleen cell homogenate equivalent to 100×10^6 spleen cells	3	3/3	$4\ 3\pm0\ 3$ $0\ 05>p>0\ 02$	4 8
100×10^6 spleen cells	10	10/10	$3\ 6\pm0\ 2$ $0\ 05>p>0\ 02$	4 7
50×10^6 bone marrow cells and 50×10^6 thymus cells	8	8/8	$3\ 9\pm0\ 1$ $0\ 05>p>0\ 02$	4 7
Lymph node cells 100×10^6	8	8/8	$4\ 6\pm0\ 2$ $p<0\ 001$	4 0
None (controls)	10	10/10	$3\ 4\pm0\ 2$	4 7

of the circulating casein antibodies was performed as stated by *Ebbesen* (1971) with minor modifications A gel containing 4 parts of 2 per cent agarose and one part of 5 per cent casein solution had the thickness set at 1 mm. Wells of 2 5 mm were filled three times with 3 µl mouse serum, obtained at autopsy and pooled for each group Incubation was performed in moist chamber until the serum was absorbed into the gel and thereafter in paraffin oil for 3 days After washing for 3 days in saline the gel was dried and stained with Coomassie brilliant blue R and the diameter of the precipitate rings measured This testing was repeated 3 times

RESULTS

All animals had developed systemic amyloidosis with the usual pattern of distribution the amyloid was most abundant in the spleen, to a lesser extent in the liver and only sparse in the kidneys The groups of animals having received spleen cell homogenate, bone marrow and thymus cells, and spleen cells in addition to casein treatment appeared to have developed the same degree af amyloidosis as the control mice having received only casein The mice having received lymph node cells contained a significantly greater amount of amyloid than the controls (Table 1)

The amount of circulating casein antibodies appeared to be nearly the same in all groups of mice except that it possible may be smallest in the mice that received lymph node cells (Table 1)

DISCUSSION

In the present experiment it was expected that transfer of intact living lymphoid cells could prevent the break down of the immune apparatus which is thought to be of pathogenic importance in the development of amyloidosis (*Teilum* 1964) However, no such effect was found, in contrast, transfer of lymph node cells caused an accelerated amyloid formation

Acceleration af casein-induced amyloidosis has been observed under many different conditions (*Christensen* 1963)—most of them conditions under which a decreased immune reactivity and a decay of lymphoid cells are prominent features cortisone treatment (*Teilum* 1952), nitrogen mustard treatment (*Teilum* 1954), X irradiation (*Christensen & Hjort* 1959), thymectomy and X-irradiation (*Ranløv* 1966), numerous cytotoxiae (*Hardt* 1971a) and thymectomy of newborn (*Ebbesen* 1971) Amyloidosis could be a result of a release of a factor (amyloid inducing factor) from decaying T lymphocytes which induces the macrophage to form the amyloid (*Hardt* 1971b) The present result could then be due to an increase of decaying cells as a result of the transfer of the lymphoid cells

The lymph node cells however which like spleen and thymus cells are known to home for their organ of origin after transfer (*Lance & Taub* 1969) were very effective especially when compared with the spleen cells This

point to an important role of the lymph node cells in amyloid formation as earlier observed by *Ebbesen et al* (1969) who by electron microscopy, found amyloid fibrils in the lymph nodes from mice with plasma cell neoplasms whereas no amyloid could be found by light microscopy using alkaline Congo red and crossed polars This may indicate that amyloid fibrils are produced *in the lymph* nodes and from there transported to the spleen (*Ebbesen* 1971)

The slight effect of the spleen cell homogenate compared to the ineffective spleen cells could be due to a non specific stimulation of the immune apparatus by the nucleo proteins (see Nucleic Acids in Immunology 1968) as a stronger immune response increases the amyloid formation (*Janigan & Druet* 1966)

Amyloid formation seems to occur independent of the level of circulating antibodies to casein (*Giles & Calkins* 1958 *Willerson et al* 1969, *Ebbesen* 1971) Hence any pathogenic importance cannot be attached to the fact that the group receiving lymph node cells seemed to have a slightly lower antibody level than the other groups

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This work was supported by grants from *Daell Fonden P Carl Petersens Fond* and the *Danish League Against Rheumatism*

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Acta path microbiol scand Section A, *80*, 471–476, 1972

STUDIES ON CASEIN-INDUCED AMYLOIDOSIS IN MICE WITH CONGENITAL APLASIA OF THE THYMUS

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The mouse mutant nude suffers a congenital aplasia of the thymus, which results in a nearly total lack of thymus dependent lymphoid cells In spite hereof amyloidosis was induced in nude mice with prolonged casein stimulation The development of amyloidosis exhibited the usual pattern of distribution, but the induction time was significantly shorter in nude mice than in normal littermates No circulating casein antibodies were found in nude mice during the development of amyloidosis The possible role of tolerance as a pathogenetic mechanism in amyloidosis is discussed

The formation of amyloid in experimental animal models as well as in man is generally believed to be due to a dysfunction of the immune apparatus Whereas the humoral part of the immune functions seems intact (*Willerson et al* 1969) Recent research into the field of amyloidosis indicates that disturbances in cell mediated immune mechanisms might be of pathogenetic importance for the development of experimental amyloidosis (*Ranløv & Jensen* 1966, *Cathcart et al* 1970 1971 *Hardt & Claesson* 1971 and 1972)

During the development of casein induced murine amyloidosis a marked stimulation, proliferation and subsequent decay of the thymus dependent lymphoid cell population has been observed (*Hardt & Claesson* 1972 1971b *Claesson & Hardt* 1972) In order to further evaluate the role of the thymus dependent cell population in amyloidosis, the effect of casein treatment of mice homozygos for the mutation nude with congenital aplasia of the thymus was studied

MATERIALS AND METHODS

Mice A stock of mice carrying the mutant nude (nu/nu) and their normally haired littermates (nu/?) were studied The mice were born and bred under specific pathogen free conditions* After arrival to our laboratory the animals were kept under clean conditions and fed sterilized mouse pellets All mice were male and 8 weeks old at the beginning of the experiment

Antigen Sterilized casein was used in a 5 per cent solution in 0 25 per cent NaOH The casein was sterilized in a 10 MeV linear electron accelerator at EAC Research Establishment, Risø The casein solution was given daily subcutaneously for 10 days 30, 40 days and 55 days

Circulating antibodies to casein Aliquots of 15 microlitres serum from individual mice were tested

Received 15 xii 71

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* Obtained from Laboratory Animals Breeding and Research Centre Gl Bomholtgård, Laven, Denmark

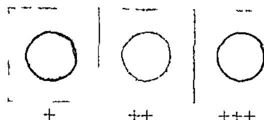


Fig 1 Various degrees of precipitating antibodies to casein scores from + → + + +

for the presence of anti casein antibodies using a *modified Mancini technique (For further details see Claesson & Hardt* (1972b)) The reaction was quantitated by visual inspection of individual areas of precipitation (see Fig 1) and scores were estimated from the weakest [+] to the strongest [+ + +] reaction as indicated in Table 1

Histology The day after the last casein injection the animals were bled and killed in ether Spleen liver kidneys mesenteric lymph node and axillary lymph nodes (draining the sites of injections) were fixed in neutral formalin and embedded in paraffin separately The mice were carefully examined for the presence of thymus tissue Sections were cut 5 microns thick and stained with haematoxylin eosin methyl green pyronine alkaline Congo red and the P A S stain Amyloid was identified by *its morphology and by its birefringence with Congo* red under crossed polars

RESULTS

No thymus was found in any of the nu/nu mice after examination of the mediastinal region, whereas the thymus was present in all nu/? littermates Histologically, the lymph nodes of the nu/nu mice were characterized by depletion of lymphoid cells in the para cortical as well as in the cortical areas (Fig 2) In the spleens of the nu/nu mice it was impossible to differentiate between white and red pulp and a clear cut periarteriolar area could not be identified (Fig 3) The mice tolerated the casein injections very well as only one out of 10 succumbed during the period of treatment with signs of wasting The remainders rather gained in weight as compared to the uninjected nu/nu mice

During the casein injections the axillary lymph nodes—which were regional to the site of injections—did not change *but* remained histologically identical to the unstimulated ones Fig 4 shows an axillary lymph node from a nu/nu mice which had received 55 *injections* of casein Still no signs of stimulation appeared as compared to the lymph node from a nu/? which had received 10 injections only (Fig 5) The spleens from nu/nu mice showed signs of stimulation and after 30 injections of casein a well developed white pulp was seen At this stage—however —as well as later on no signs of germinal centre activity was found (Fig 6) In the *stimulated spleen of the nu/nu* mice the typical depletion of lymphoid cells in the periarteriolar sheet of the white pulp was con

TABLE 1 *Histological Findings and Humoral Antibody Response to Casein in Nu/Nu and Nu/? Mice During Casein Stimulation*

Number of animals		Number of casein injections	Perifollicular pyroninophilic reaction in the spleen		Amyloid incidence in the spleen		Serum antibodies to casein	
nu/nu	nu/?		nu/nu	nu/?	nu/nu	nu/?	nu/nu	nu/?
2	2	10	moderate	strong	0/2	0/2	0 0	0 0
2	2	30	moderate	strong	0/2	0/2	0 0	0 +
2	2	40	weak	strong	2/2	0/2	0 0	+ +
3	3	55	absent	moderate	2/3	1/3	0 0 0	+ + + + + + + +



Fig 2 Axillary lymph node from an untreated nu/nu mouse Note the nearly total depletion of lymphocytes in paracortical as well as cortical areas H & E × 84

stantly present Only a weak perifollicular pyroninophilia was observed In contrast to this, the nu/? mice, developed a striking perifollicular pyroninophilic proliferation during the course of treatment, and numerous germinal centres were observed

Amyloid deposits were found in both groups of mice as indicated in Table 1 The amyloid appeared earlier in the nu/nu mice but in both groups it was found in the typical perifollicular position, and in some of the nu/nu mice undergoing prolonged stimulation the amyloid tended to obliterate the red pulp leaving the white pulp as mere islets in the splenic stroma (Fig 7) As shown in Table 1 only 1 out of 5 nu/? mice in contrast to 4 out of 5 nu/nu did develop amyloidosis In the nu/nu group, amyloid was also identified in the liver, in one of these animals, amyloid deposits were seen also in the kidneys Table 1 further shows the humoral antibody response to casein as evaluated by immuno-diffusion methods No antibody to casein was found in the nu/nu mice, while their heterozygote counterparts all showed antibody production after 30 injections of casein

DISCUSSION

In agreement with *de Sousa et al* (1969), we found in the non-stimulated, nu/nu mice that the thymus-dependent areas of the lymph nodes and spleens were severely depleted of lymphocytes Furthermore, we observed a marked depletion of cortical lymphocytes and a poorly differentiated spleen architecture The prolonged treatment with casein did not alter the features of the lymph nodes, but the casein treatment did lead to the development of a well differentiated red and

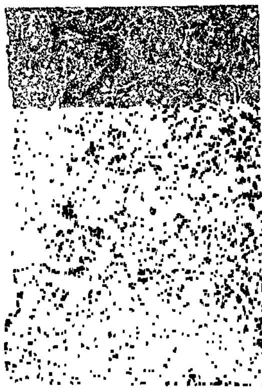


Fig 3 Spleen from an untreated nu/nu mouse Note the poorly differentiated splenic pulpa. H & E × 84

white splenic pulp. Thus it might be more relevant to assume a general sensitivity of lymphoid organs to the lack of thymus, than to claim the presence of specific thymus dependent areas as done be (*Parott et al* 1966) This suggestion is further supported by the observation of paracortical as well as cortical lymphocyte depletion in neonatally thymectomized mice (CBA strain) undergoing wasting disease (*Jorgensen* & *Claesson*)

Even after 55 injections of casein the nude mice failed to produce humoral antibodies to casein This is in line with the findings of *Wortis* (1971) who found a reduced plasma level of immunoglobulins and a severely reduced response to sheep RBC in the nude mouse The amyloid formation, however, took place regardless of the lacking circulating antibodies to casein Similarly, *Clerici et al*



Fig 5 Axillary lymph node from a nu/? mouse having received 10 injections of casein Note the germinal centres as well as a diffuse infiltration of lymphocytes in cortical and paracortical areas H & E × 105



Fig 4 Axillary lymph node from a nu/nu mouse, having received 55 injections of casein No signs of stimulation are seen H & E × 84

(1965) found mice with acquired tolerance to casein (as judged by the absence of circulating anti-casein antibodies) capable of developing amyloidosis when chronically treated with casein

Experimental procedures known to accelerate the formation of amyloidosis namely application of cytotoxics (*Teilum* 1954, *Hardt* 1971), irradiation (*Christensen* & *Hjort* 1959) and thymectomy (*Ranløv* 1966 *Clerici et al* 1969, *Ebbesen* 1971) are conditions under which a severe reduction in the number of immuno competent cells are regularly observed The nude mouse—with an inherent depletion of immuno competent ... faster and to a

experiments may in fact support the theory



Fi   a mouse hav ng received 30   casein. Note the well d fferent ated splc t pulps with well developed lymphoid follic les The periarteriolar deplet on of lymphocytes is shown (arrows) H & E × 84

of *Cathcart et al* (1970) that specific cellular tolerance could play a role in the pathogenesis of amyloidosis as tolerance is facilitated wl en the number of immunocompetent cells are reduced (*Taylor* 1964)

However according to *Hardt & Claesson* 197? the tolerance observed by *Cathcart et al* 1971) could be merely an associated phenomenon to the general and severe re duction in the number of thymus dependent lymphocytes

It has been postulated previously by us that a factor from the decaying thymus dependent lymphocytes together with the ntigen could induce the macrophages to f rm the amyloid

Th is hypothesis could also be valid in the n ide mo i e as even in this mouse some thymic influer ce seems to be operating foe tally and neonatally (*Pantelouris & Hai* 1970) In fact it has been shown that lymphocytes are present in the nude mice, whicl carry the O antigen specific for thymus de pendent lymphocytes (*Raff & Wortis* 1970) and which are capable under germ free con ditions to reject allogeneic skin grafts (*Ry gaard et al* 1971)

We wish to thank cand med vet B H Eriksen The Danish Atom c Energy Commission Research Establishment Riso for sterilizing the casein solu tion This work was supported by a grant from the *Danish League Against Rheumatism*

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fi   arrows) in the spleen
of   ·   se having received 40 injections
               H & E × 210

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Acta path. microbiol scand Section A 80, 477–486, 1972

RELEASE OF ANTIGEN-BINDING CELLS FROM THE SPLEEN INTO THE BLOOD

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The splenic release of lymphocytes specifically engaged in an immune response was quantitated in guinea pigs during a secondary response to sheep red blood cells (SRBC). The number of cells forming rosettes with SRBC was compared in splenic venous and arterial blood. The splenic veno-arterial difference in the number of rosette forming cells (RFC) was compared in normal guinea pigs and at different intervals after a booster dose of SRBC, given 3 weeks after a primary dose. In normal guinea pigs there was no release of RFC from the spleen. A small release was found 3 weeks after a primary dose of SRBC. After a booster dose the splenic veno-arterial difference in the number of RFC increased to a maximal value after 6 days. Simultaneously, the number of RFC was increasing in both the spleen and the blood. The results clearly indicate that the spleen releases lymphocytes with specific antigen receptors during the secondary immune response to SRBC.

A release of splenic lymphocytes into the blood normally occurs in the guinea pig as judged by cellular counts of splenic afferent and efferent blood (*Sandberg* 1970). Since this cellular migration is influenced by immunization (*Sandberg* 1972), it is linked to the immunological activity of the spleen.

The presence of antibody forming or antigen binding cells in the blood has been demonstrated by various methods (*Gunderson et al.* 1962, *Hulliger* & *Sorkin* 1963, 1965, *Landy et al.* 1964, *Kearney* & *Halliday* 1965, *Sorkin* & *Landy* 1965, *Biozzi et al.* 1967, *Halasa* 1968, *Hiramoto et al.* 1968, *Simons* & *Fitzgerald* 1969, *Rabin* & *Rose* 1970). However, a direct demonstration and quantitation of their release from the spleen has not been reported. The present investigation is an attempt to prove such a splenic release of cells specifically involved in an immune response to sheep red blood cells (SRBC).

The technique used to trace the cells involved in the response to SRBC is based upon the existence of antigen specific receptors on the cell surface. The cells are identified by the specific adherence of the antigen, so-called immunocytoadherence (*Nota et al.* 1964). Using heterologous erythrocytes as antigen (*Nota et al.* 1964, *Osipova* & *Karasik* 1964, *Zaalberg* 1964) the adherence of erythrocytes to mononuclear cells with specific receptors gives rise to what has been named under somewhat different conditions "rosettes" (*Biozzi et al.* 1967) or "clusters" (*Zaalberg* 1964).

In order to detect a splenic release of cells specifically engaged in the immune response, the number of rosette-forming cells (RFC) was compared in afferent and efferent splenic

Received 15.xii.71

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blood and related to the number of RFC in the whole spleen during a secondary immune response to SRBC

MATERIAL AND METHODS

Animals

Male guinea pigs were used for the quantitation of rosette forming cells (RFC) in the afferent and efferent splenic blood and in the spleen Normal animals and immunized animals investigated at different intervals after a secondary immunization with SRBC were compared The weight of the immunized animals averaged 283 g at the first injection of SRBC and 432 g at the second injection The normal animals weighed 409 g as a mean

Immunization

Sheep red blood cells suspended in Alsever's solution were washed four times and resuspended in 0 9 per cent saline A 10 per cent suspension of SRBC was used as antigen and injected intraperitoneally in a dose of 2 5 ml (corresponding to 5×10^9 erythrocytes) After 3 weeks the animals were given a second injection with the same dose The animals were investigated 2, 4 6 and 8 days after the second injection (17, 13 41 and 20 animals, respectively) Primary immunized controls (21 animals) were investigated 3 weeks after the first injection of SRBC Blood from the same sheep (the National Bacteriological Laboratory, Stockholm) was used for both primary and secondary immunizations as well as for the RFC test (see below)

Blood samples

The sampling of blood has been described in detail previously (*Sandberg* 1970) The sampled amount of venous blood was chosen as small as possible in order to avoid unnecessary disturbance the haemodynamics which might influence the flow of blood in the splenic artery A sample of 25 µl of blood was used to determine the number of mono and polynuclear cells per µl and 200 µl of blood was collected from the splenic vein and artery, respectively to determine the number of RFC

The 200 µl blood samples were transferred into 3 ml tubes containing 1 ml of 4 per cent dextran (Dextran T 250 M W 250 000 Pharmacia Uppsala Sweden) and one drop of heparin (Heparin® Vitrum, Stockholm 5 000 IE/ml) The tubes were placed at 4° C for 40 min and the erythrocytes allowed to sediment The leucocyte rich supernatant was transferred to another tube to which 1 ml of cold buffer (containing ⅓ volume of 0 15 M phosphate buffer pH 7 25 and ⅔ volume of 0 15 M NaCl) was added This tube was then centrifugated at 17 g for 4 min which resulted in the sedimentation of additional erythrocytes The leucocytes in the supernatant were then washed twice in cold buffer After the last centrifugation the pellet of cells was resuspended in 0 2 ml of the buffer, giving a concentration of approximately 1 500 mononuclear cells per µl 40 µl of a 0 3 per cent suspension of SRBC was then added The cells were allowed to sediment in the tube (8 × 55 mm) at 4° C for 3 hr

Spleen Cell Suspensions

The individual spleens were cut into small pieces which were gently pressed by a glass piston while being rinsed with a cold buffer solution (described above) The suspension was then passed through nylon nets to split up aggregates of cells (finest net had a mesh size of 80 µm) Slight underpressure was used to facilitate the passage through the nets The total number of mononuclear cells in the suspension was determined The cells were then washed twice in buffer and their concentration adjusted to 6×10^6 mononuclear cells per ml To 1 ml of this suspension in a 3 ml tube (8 × 55 mm) 30 µl of a 10 per cent suspension of SRBC was added The tubes were placed at 4° C overnight and the cells allowed to sediment

Determination of the Number of RFC

The cells were resuspended by turning the tubes upside down at 30 revolutions per minute for 2 min A white cell count was performed on each sample The suspension was searched for the occurrence of RFC by examination in a Bürker chamber at 400 × magnification At least 10 000 spleen mononuclear cells and at least 5 000 mononuclear cells from each blood sample were examined from each animal Mononuclear cells with 5 or more sheep erythrocytes firmly attached were registered as RFC Rounded clusters of sheep erythrocytes which always were of uniform size were also counted They presumably consisted of one layer of erythrocytes around a white cell The number of RFC per 10^4 cells was calculated

Effect of the Amount of SRBC on the Quantitation of RFC

To ensure an optimal ratio between sheep erythrocytes and leucocytes a technical experiment was performed Various amounts of SRBC were added to different leucocyte suspensions and the number of RFC was examined

Suspensions of spleen cells were prepared from immunized and from non immunized guinea pigs as described above From each animal the suspension was divided into different tubes and various amounts of SRBC (0–40 µl of a 10 per cent

suspension) were added After sedimentation at 4° C the number of RFC was determined

Blood samples were taken from the splenic vein of immunized guinea pigs The blood leucocyte suspensions from one animal were prepared as described above and then pooled and again divided into separate tubes From 0 to 60 µl of a 0 3 per cent suspension of SRBC were added to the different tubes The cells were allowed to sediment at 4° C and the number of RFC was counted

Experiments on Rosette forming Macrophages

Rosettes formed by macrophages (false rosettes) are probably due to the attachment of SRBC to cytophilic antibody on the surface of macrophages or monocytes (for references see *Bio t et al* 1967) Normal serum is said to inhibit the formation of false rosettes when added before (*Lokaj* 1970) as well as after the fixation of cytophilic antibodies to the macrophages (*Jonas et al* 1965 *Kossard & Nelson* 1968) The following experiments were made in order to study the possible occurrence of false rosettes due to the presence of cytophilic antibody in the present experiments

1 Incubation of Normal Mononuclear Cells with Normal or Immune Serum

Spleen cell suspensions from normal guinea pigs were mixed with serum from normal or immunized guinea pigs The immune serum was prepared from animals found to have a high content of splenic RFC 6 days after a second immunization with SRBC To 1 5 ml of each cell suspension 75 µl of guinea pig serum was added The cell suspensions were then incubated at 37° C for 30 min Four experiments were performed

a) incubation with immune serum
b) incubation with normal serum
c) incubation with immune serum followed by washing twice and a second incubation with normal serum
d) incubation without serum

After the incubation the cells were washed twice SRBC added and the number of RFC determined as described above

In order to confirm the presence of antibodies cytophilic for macrophages in the serum from immunized animals and to study the effect of normal serum on the fixation of such antibodies to macrophages the same experiment as described above was performed with suspensions of peritoneal macrophages from non immunized guinea pigs

Blood cell suspensions prepared from samples from the splenic vein were incubated at 37° C for 40 min with immune serum (50 µl of serum was added to the tubes with dextran) Control suspensions were incubated without serum The subsequent procedures were made as described above with quantitation of the RFC

2 Incubation of Cells from Immunized Animals with Normal Serum

a Normal serum added together with SRBC After the blood and spleen cells had been washed, normal guinea pig serum which had been adsorbed with SRBC was added to the suspensions 20 µl was added to the spleen cells and 40 µl to blood cells SRBC were added and the number of RFC was determined and compared to the number in samples of the same suspensions without serum added

b Normal serum added before SRBC 75 µl of normal serum (adsorbed with SRBC) was added to 1 5 ml of spleen cell suspensions from immunized animals and 50 µl of normal serum was added to the blood cells in dextran After incubation for 30–40 min at 37° C the cells were washed in the usual way and the number of RFC determined and compared with values obtained when no incubation was performed

Statistical Analysis

The differences in content of leucocytes or RFC per µl or ml of blood in splenic afferent and efferent blood were calculated in the individual animals and they were analysed statistically by the Student's *t* test

RESULTS

Experiments on the Technique

Effect of the amount of SRBC on the quantitation of RFC No RFC were obtained in the absence of SRBC but a small number of mononuclear cells was found with one or two adhering autologous erythrocytes When suspensions of spleen cells were tested with various amounts of SRBC, the result showed an increasing number of RFC with increasing amounts of SRBC up to a point where no further increase was obtained (Table 1) In fact the addition of further amounts of SRBC tended to inhibit rosette formation From the results it was concluded that an amount of 30 µl of a 10 per cent suspension of SRBC was optimal for identifying the RFC in the suspension of spleen cells

The results of the same approach regarding the RFC in the blood are shown in Table 2 The highest number of RFC was obtained with 40 µl of a 0 3 per cent suspension of SRBC

TABLE 1 *Effect of the Amount of Sheep Erythrocytes on the Quantitation of RFC in the Spleen*

Animal no	Volume (µl) of a 10% SRBC suspension added					
	0	5	10	20	30	40
			Immunized animals			
1	0	25	29	68*	–	–
2	0	–	46	105*	89	–
3	0	–	22	47*	46	–
4	–	–	48	275*	146	93
5	–	–	8	23	37*	28
6	–	–	13	17	27*	26
7	–	–	57	109	130*	120
			Non immunized animals			
8	0	23	33*	16	–	–
9	0	–	6	13	17*	–
10	0	–	6	9	16*	
11			–	19	32*	28

Number of RFC per 10^4 spleen cells
* The highest number in each animal

TABLE 2 *Effect of the Amount of Sheep Erythrocytes on the Quantitation of RFC in the Blood*

Animal no	Volume (µl) of a 0 3% SRBC suspension added						
	0	10	20	30	40	50	60
1		3	0	63	120*		
2	–		32*	29	32*		
3		8	18	22	51*	–	–
4			17	39	101	227*	
5	–				241*	164	
6	0	–		92	111*	92	
7	–			28	39*	17	37
8				4	7	13	18*
9				21	38*	13	10

Splenic venous blood of hyper immunized animals
Number of RFC per 10^4 blood mononuclear cells
* The highest number in each animal

Rosette forming macrophages Incubation of normal spleen cells with immune serum at 37° C resulted in a distinct increase in the number of rosettes (Table 3) Contrarily, incubation with normal serum had no effect The appearance of false rosettes could not be inhibited by simple washing of the cells after incubation with immune serum However, an after incubation with normal serum at 37° C abolished the increase in rosette formation found after incubation with the immune serum It is evident that a great number of cells in the spleen have the ability to form false rosettes under proper conditions No increase of RFC could be found in the blood after incubation with immune serum

No rosettes were formed by peritoneal cells after incubation with normal serum or without serum, but incubation with immune serum resulted in the appearance of a great number of rosettes A second incubation with normal serum completely inhibited this formation of rosettes (Table 3)

Incubation with normal serum of spleen or blood cells from animals 6 days after a second immunization with SRBC had no effect on the number of rosettes (Table 4) The interpretation must be that there was no significant number of rosettes formed by macrophages since treatment with the normal serum should have inhibited the formation of false rosettes as shown above

RFC in the Spleen of Normal and Immunized Animals

The spleen of normal male guinea pigs contained approximately 14 RFC per 10^4 mononuclear cells or 5×10 RFC per spleen This basal level was significantly elevated ($p<0\,01$) 3 weeks after the first injection of SRBC, when the number of RFC per 10^4 cells was more than doubled This was not due to an increase of the background level with age, since the weight of the selected normal animals was the same as that of the animals in the preimmunized group The number of RFC was further increased after the secondary immunization The highest number of RFC per 10^4 cells and the highest total number of RFC per spleen was found after 8 days, although the RFC were almost as numerous after 6 days (Fig 1 and Table 5)

The number of mononuclear cells per spleen was not significantly altered by the immunization and averaged 244×10^8 (124 animals)

TABLE 3 *Effect of Incubation of Normal Spleen Cells, Peritoneal Cells or Blood Leucocytes with Immune Serum on the Rosette Formation*

		immune serum	immune serum + normal serum	normal serum	no serum
Spleen cells	(6)*	147 ±29	–	–	20 ±4
	(10)*	85 ±26	32 ±7	27 ±3	25 ±3
Peritoneal cells	(4)	300 ±59	2.5 ±1.7	0	0
Blood cells	(6)	2.8 ±2.1	–	–	2.3 ±0.8

Controls were incubated without serum, with normal serum, or immune serum followed by normal serum. Mean number of RFC per 10^4 cells ± standard deviation of the mean Number of experiments within brackets

* Two different groups of experiments

TABLE 4 *Rosette Formation after Incubation of Spleen Cells or Blood Leucocytes from Immunized Animals with Normal Serum*

	A serum added before SRBC	B* no serum	C serum added with SRBC	D** no serum
Spleen cells	135 (5) ±47	101 (5) ±52	253 (10) ±32	259 (10) ±34
Blood cells	3.5 (4) ±1.6	3.3 (4) ±1.7	4.4 (5) ±0.7	4.8 (5) ±1.7

The serum was added before SRBC (followed by washing of the cells) or simultaneously Mean number of RFC per 10^4 cells ± standard deviation of the mean Number of experiments within brackets The incubation with normal serum did not reduce the number of rosettes

* Untreated cells from same animals as in group A.

** Untreated cells from same animals as in group C

RFC in the Blood of Normal and Immunized Animals

An average number of 2 RFC per 10^4 blood mononuclear cells was found in the normal animals (splenic artery blood) This number was slightly increased 3 weeks after primary immunization with SRBC After the secondary immunization the number rose to 15 RFC per 10^4 cells on the 8th day (Fig 2 and Table 6)

Splenic Release of Leucocytes

In all the groups of animals the content of leucocytes was higher in splenic efferent than in the afferent blood (Fig 3) In the normal animals the difference in number of lymphocytes approximately 1000 cells per μl, is the same as that found earlier in untreated animals of the same weight (*Sandberg* 1970) This difference was more pronounced 3 weeks after the first injection of SRBC The second injection of SRBC caused a transient decrease to normal levels during the next few days

Splenic Release of RFC

The number of RFC per 10^4 mononuclear cells in splenic afferent and efferent blood is

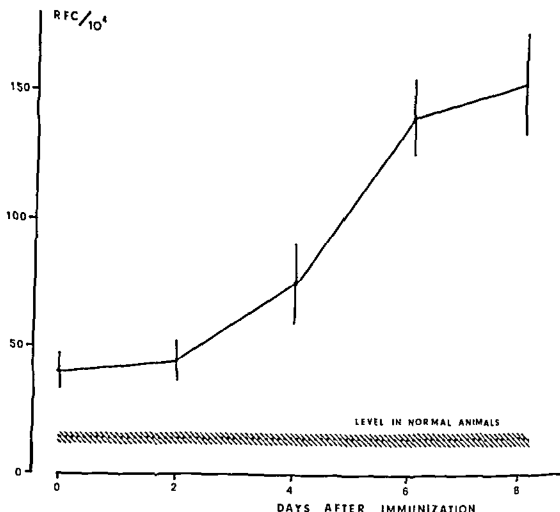


Fig 1 Number of RFC per 10^4 spleen mononuclear cells of normal guinea pigs and at various intervals after a secondary immunization with SRBC given 3 weeks after a primary immunization Mean ± standard deviation of the mean

shown in Fig 2 In normal animals no difference between afferent and efferent blood was found At 3 weeks after the first immunization the number of RFC per μl of blood from the splenic vein exceeded that from the splenic artery ($p<0.05$), suggesting a release of cells with receptors for SRBC from the spleen into the blood Two days after the secondary injection of SRBC no such difference was found, but after 4 and 6 days evidence was again obtained of a release of splenic RFC into the blood (Fig 4)

DISCUSSION

The RFC should not be considered as antibody-forming cells in the sense that they release antibody Under the conditions in this investigation, the rosettes are formed solely on the basis of the occurrence of antigen specific receptor molecules on the cell surface Such receptors have been shown to share many characteristics with immunoglobulin molecules (*McConnel et al 1969*), but their presence does not necessarily mean that the cells release antibody In fact, evidence is accumulating that at least part of the RFC and the antibody producing cells belong to different cell populations (*Gudat et al* 1970, *Wilson* 1971)

In this connection some details in the experimental design deserve discussion One is whether or not an incubation period at

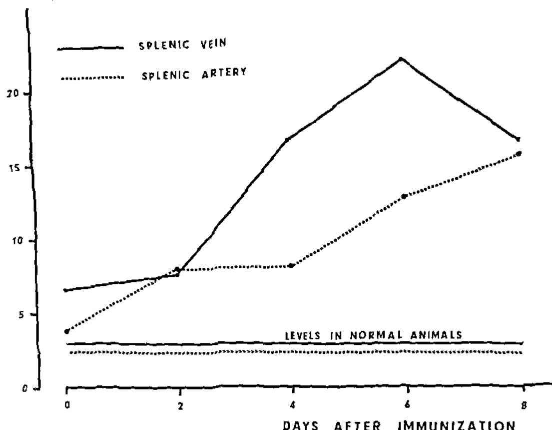


Fig 2 Mean number of RFC per 10^4 mononuclear cells from splenic arterial and venous blood of normal guinea pigs and at various intervals after a secondary immunization, given 3 weeks after a primary immunization

TABLE 5 *Total Number of RFC per Spleen in Normal and Immunized Animals*

Animals		RFC × 10^5
Normal		4 7±1 2
Days after	0	8 9±1 3
secondary	2	10 0±1 8
immunization	4	19 7±5 1
	6	36 4±4 9
	8	37 8±5 2

Mean ± standard deviation of the mean

TABLE 6 *Number of RFC per 10^4 Blood Mononuclear Cells in Splenic Venous and Arterial Blood in Normal and Immunized animals*

Animals		venous blood	arterial blood
Normal		3±2	2±1
Days after	0	7±2	4±1
secondary	2	8±2	8±2
immunization	4	16±5	8±3
	6	22±5	13±3
	8	16±5	15±3

Mean ± standard deviation of the mean

37° C is used No such incubation is needed for the detection of RFC (*Laskov* 1968), but under appropriate conditions it may increase their number (*Wilson* 1971) as well as the number of clusters (*Shearer* & *Cudkowicz* 1968) The reason for this seems to be the production of antibody by some cells during the incubation at 37° C The RFC attaching more than one layer of erythrocytes probably appear as a result of production of haemag

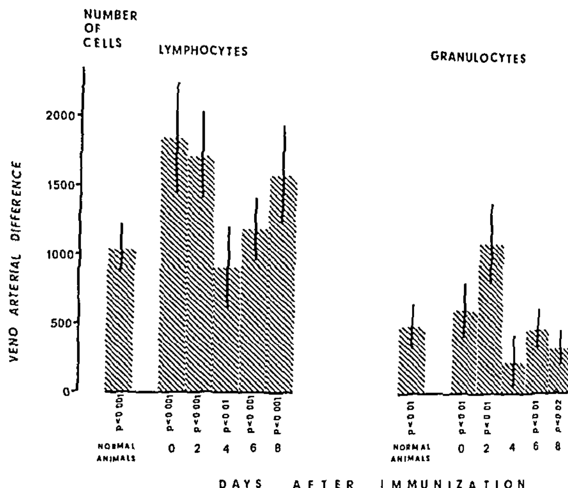


Fig 3 Difference in the number of lymphocytes and granulocytes per µl of blood from a splenic vein and artery of normal guinea pigs and at various intervals after a secondary immunization with SRBC, given 3 weeks after a primary immunization Mean difference ± standard deviation of the mean

glutinin (*Duffus* & *Allan* 1971) This may explain the finding that some rosettes formed at 37° C are instable (*Wilson* 1971) and that washing of the cells reduces the number of RFC (*Laskov* 1968), possibly by the removal of loosely bound antibodies Evidently incubation at 37° C results in the appearance of different kinds of RFC, but also rosettes formed without incubation may contain two types of cells, one of which alone represents the background RFC (*McConnel* 1971) Reference should also be made to studies on the origin of the RFC showing that some, but not all, are thymus derived (*Greaves* & *Möller* 1970)

The amount of heterologous erythrocytes in relation to mononuclear cells used for the quantitation of RFC proved to be important With too many or too few erythrocytes, rosette formation would be inhibited The inhibition of rosette formation in the presence of supra optimal amounts of erythrocytes was also observed by *Laskov* (1968) Another factor affecting the number of RFC is the size of the test tubes (*Wilson* 1971) In the present investigation, therefore, glass tubes of uniform size were used

In studies of RFC, the possible occurrence of false rosettes should always be considered In the present study, the presence of cytophilic antibody was demonstrated in sera from immunized animals It was also shown that a number of cells from the spleen could fix cytophilic antibody by *in vitro* incubation with im

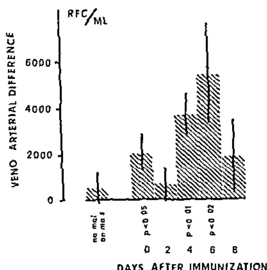


Fig 4 Difference in the number of RFC per ml of blood from a splenic vein and artery of normal guinea pigs and at various intervals after a secondary immunization with SRBC given 3 weeks after a primary immunization Mean ± standard deviation of the mean

mune serum and cause rosette formation Up to two per cent of the suspended spleen cells had this ability This is enough to affect the total number of RFC In fact, in some cases incubation of normal spleen cells with immune serum resulted in an increase in total number of RFC to a level found in immunized animals In spite of this, no detectable levels of false rosettes could be demonstrated in this experimental model among the spleen or blood cells of immunized animals

This investigation showed that a release of splenic RFC into the blood occurs after immunization with SRBC Two days after the second injection of SRBC the difference in content of RFC in splenic afferent and efferent blood was less pronounced possibly indicating an accumulation of specific cells in the spleen after antigen challenge A specific recruitment of immunologically competent cells from the circulation has been demonstrated in mice during the first days after immunization with SRBC (*Sprent et al* 1971)

In the rat, a migration into the blood of splenic cells formed in response to immunization was demonstrated by *Cannon & Wissler* (1965) They suggested that the migrating cells were derived from antibody forming cells in the red splenic pulp In the present work a migration of antigen specific cells from the spleen into the blood was directly demonstrated They constituted only a minor part of the total release of cells Their role is not clear, but some of them may belong to the pool of antibody forming cells in the blood, playing a role in total antibody production (*Hulliger & Sorkin* 1963, *Halasa* 1968) Part of them may be memory cells homing in different lymphoid organs or recirculating between blood, tissues and lymph

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This work was supported by grants from the *Swedish Cancer Society* and *Karolinska Institutet* (Reservationsanslaget)

I am indebted to Vitrum, Stockholm, for generous supply of heparin

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Acta path microbiol scand Section A, 80, 487–490, 1972

A CASE OF THYMONA IN ASSOCIATION WITH MEGAKARYOCYTOPENIA

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A case of thrombocytopenic purpura megacaryocytopenia and signs of auto immune processes (positive direct Coomb s test) is presented It was later complicated by an intracranial haemorrhage At autopsy a thymoma (microscopically a lymphoepithelioma with spindle cell predominance) was found The possible causal relationship between the two disorders is discussed

During the last decade, several reports of co existing thymoma and haematological dis orders have been published (see *Hirst & Robertson* 1967, *Goldstein & Mackay* 1969, *Zoupanos* 1970 for reviews) It has been proposed that the disorders of the thymus and the bloodforming organs were causally related

Although a single case of thymoma and erythrocytosis (*Sundström* 1972) has been observed, thymic tumours are most frequently seen, among haematological disorders, together with bone marrow hypoplasia of dif ferent kinds In most cases there is pure red cell agenesia (erythroblastopenia) One of the latest reports of this kind was presented by *Hamilton & Conley* (1969) Sometimes aplastic anaemia with panaplasia (pancytopenia) is seen together with a thymoma (*Korn et al* 1967, *Talerman & Amigo* 1968) A case of thymoma and lymphopenia (*Braunsteiner et al* 1968) has been published *Friedman* (1967) has mentioned (in Table 2), without further comments, a case of thymocarcinoma and thrombocytopenia The present report deals with a case with severe thrombocytopenic purpura, megacaryocytopenia, signs of auto immune processes and a thymoma This seems to be the first case hitherto observed

CASE REPORT

Clinical Data

A woman aged 90 (S R, 800909) was admitted to the hospital in March 1971 During the last three years she had been treated in the out patient department with B_{12} injections (1 mg every two month) due to a suspected but not proved, pernicious anaemia She had no other treatment For the last few days before admission she had suffered from epistaxis, weakness, dizziness and she had bruised easily

Positive clinical signs at the first physical examination were widespread petechiae, echymoses and submucous haemorrhages systolic mumur grade II III over the apex, heart rate 95 per min There were macroscopic haematuria and melaena

Initial laboratory findings were haemoglobin concentration 7 0 g/100 ml, haematocrit 21 per cent red cell count 2 8 millions/mm³, reticulocytes 0 6 per cent white blood cell count 4 900/mm³ with norma[illegible]
less than [illegible]
haptoglobin [illegible]
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in serum were 5 6 and 3 8 g/100 ml, respectively A bone marrow aspirate (smear) showed a normal myelopoiesis, but, considering the continous bleeding, a rather low activity of the erythropoiesis In an embedded bone marrow aspirate there were strikingly few megacaryocytes In the serum of the patient, irregular iso-antibodies against several test donor erythrocytes were found using enzyme or indirect antiglobuline (Coomb's) technique The red cells of the patient were found to be coated with complement and auto-antibodies of IgG and IgM types (positive direct Coomb's test) The possibilities of auto antibodies against other cells or cell constituents were not studied

Unfortunately, no chest x ray examination was performed this time A normal chest x ray was obtained in Oct 1965

The patient had blood transfusions and was treated with prednisolone in doses of 3 mg/kg/day However, the thrombocytopenia was not affected by the treatment and after a period of diffuse haemorrhagic diathesis and signs of intracranial haemorrhage she died 14 days after admission to hospital

Autopsy Findings

Autopsy was performed four days after death Petechiae were found in the skin of the lower legs subcutaneous haemorrhages on the left knee, the hands and arms and below the left eye The coronary arteries (weight of heart 350 g) showed moderate arteriosclerotic changes In the mediastinum, a tumour the size of a mandarin was found located outside the pericardium but in connection with its upper front surface The tumour was well defined and supplied with a fibrous capsule The cut surface was yellowish white and showed several small haemorrhages No metastases were found In both lungs there were small areas of atelectasis and some oedema The left kidney (weight 120 g) showed moderate reduction of the cortex the right kidney (weight 165 g) was normal The right renal pelvis, the urinary bladder and the stomach were sites of multiple submucous haemorrhages In the right parietal lobe a central haemorrhage the size of a hazelnut was found (weight of brain 1 110 g)

Several specimens from the tumour were stained

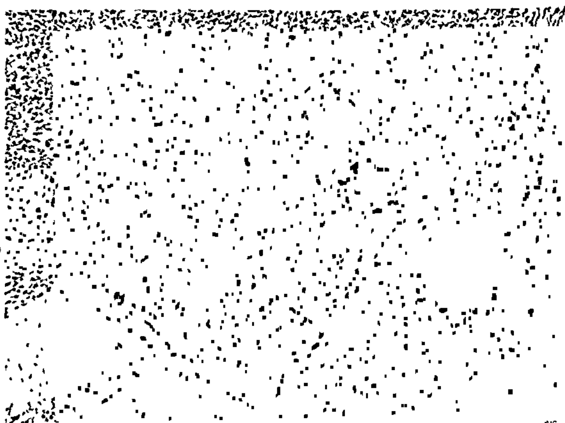


Fig 1 Microscopic appearance of a thymoma from a woman aged 90 with severe thrombocytopenic purpura megacaryocytopenia and signs of auto immune processes The tumour was mostly of spindle cell predominance type but the micrograph above shows one of the areas with mixed epithelial appearance and strands of connective tissue (X 180 vG)

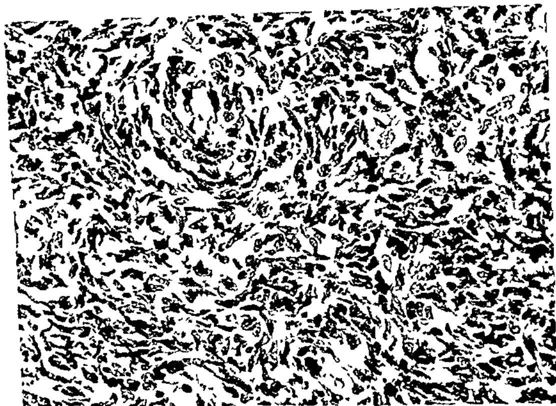


Fig 2 Micrograph from the same thymoma showing spindle shaped cells in whorls (X 400, v G)

according to van Gieson, with haematoxylin eosin, periodic acid Schiff's reagent (PAS), methyl green pyronine, toluidine blue, and silver impregnated according to Laidlaw The tumour was mostly built up by cords and strands of spindle shaped epithelial cells with elongated oval nuclei and sparse cyto plasm which was weakly positive to eosin and negative to PAS A few accumulations of more typical epithelial cells were seen Lymphocytes of ordinary appearance were found interspersed with in the otherwise homogenous tumour tissue There were also a few scattered aggregates of lymphocytes No Hassall's corpuscles were observed. Abundant connective tissue strands were interspersed within the tumour tissue No signs of cross striation were seen Reticulin fibres were abundant in the connec tive tissue strands but otherwise sparse The inter cellular substance was in some places positive to PAS No plasma cells eosinophilic cells, giant cells or mast cells were seen No germinal centres were found There was no excess of blood vessels but haemorrhages could be seen near the well-developed fibrous capsule The histopathological picture was considered compatible with a thymoma (Figs 1 2)

DISCUSSION

The thymoma observed in the present case was classified as a lympho-epithelioma with spindle cell predominance (*Galy* & *Renault* 1969) This type of thymoma is that most frequently (65 70 per cent) found in cases of concomitant thymoma and pure red cell agenesia (*Schmid et al* 1965, *Hirst* & *Robertson* 1967) In order to explain the pos sible relationship between thymoma and pure red cell agenesia two general theories have been proposed (see *Goldstein* & *Mackay* 1969 for review) According to the first one, the thymus produces an agent which depresses the production of red cells in the bone marrow (*Hirst* & *Robertson* 1967) Several authors have tried to demonstrate the suspected marrow depressor using different methods, but so far, most of them have been unsuccessful *Field et al* (1968), however, demonstrated in the serum of a patient with

thymoma, Hodgkin's disease and pure red cell agenesia a factor with suppressive effect on the erythropoiesis, the data presented seemed to exclude the possibility of an antibody The second theory concerning the relationship between thymoma and pure red cell agenesia deals with the possibility that the thymoma is part of an auto-immune reaction affecting the erythropoiesis by a production of auto-antibodies against either erythropoietin or erythroblasts (*Anderson & Ladefoged* 1963, *Dameshek et al* 1967, *Krantz & Kao* 1967) *Jepson et al* (1968) demonstrated a gamma globulin factor (located in the IgG fraction) in plasma from patients with thymoma and pure red cell agenesia

In the present case it is tempting to assume that the thymoma and the thrombocytopenia are interrelated in a similar way as the well documented cases of thymoma and pure red cell agenesia The positive direct Coomb's test favours the second explanation presented above Auto antibodies against megacaryocytes might have been produced thus causing the hypoplasia of megacaryocytes in the bone marrow aspirate and the thrombocytopenia observed However, the deficiency of megacaryocytes combined with, considering the continous bleeding, the unexpected low erythropoietic activity and the low proportion of reticulocytes in the blood could point to an early aplastic anaemia (pancytopenia)

As in many other cases of thymoma and auto immune disorders it is difficult to determine whether the thymoma is primary or secondary in relation to the auto immune process

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Acta path microbiol scand Section A 80 491-500, 1972

CIGARETTE SMOKING IN RELATION TO CORONARY AND AORTIC ATHEROSCLEROSIS

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The relationship of cigarette smoking to coronary and aortic atherosclerosis was analysed in two autopsy series one comprising 84 men aged 20–77, who had died by violence and another comprising 94 men aged 30–78, who had died suddenly from coronary heart disease The extent of atherosclerosis was assessed by measuring the surface areas of atherosclerotic lesions by point counting technique In the series of violent deaths, both the coronary and aortic atherosclerosis was more severe in cigarette smokers than in nonsmokers As could be expected in the series of men who had died from coronary heart disease and generally had an advanced atherosclerotic involvement of coronary arteries, the severity of coronary lesions showed no relationship to smoking habits In the series of coronary deaths however, aortic atherosclerosis tended also to be more extensive in cigarette smokers than in non smokers

According to numerous epidemiological investigations, both the morbidity and the mortality from coronary heart disease are higher in smokers, particularly in cigarette smokers, than in nonsmokers (recent reviews e q *Fletcher & Horn* 1970, *Report of Inter Society Commission for Heart Disease Resources* 1970 *Seltzer* 1970) It has been suggested that cigarette smoking precipitates severe acute manifestations of coronary heart disease without having any direct effect on the atherosclerotic process (*Doyle et al* 1964) This assumption was based on combined results obtained in Framingham and Albany s studies in which the incidence of myocardial infarction was found to be higher and the mortality from coronary heart disease greater in cigarette smokers than in nonsmokers, while cigarette smoking was not found to be associated with the incidence of angina pectoris

Only a few patho-anatomical investigations on the relationship of smoking to atherosclerosis have been published and the results are in part contradictory *Wilens & Plair* (1962) and *Sackett et al* (1968) found aortic atherosclerosis to be more severe in cigarette smokers than in nonsmokers Sackett et al showed that the degree of atherosclerosis correlated with the average number of cigarettes smoked per day and with the duration of smoking *Auerbach et al* (1965) observed that coronary atherosclerosis was more severe in cigarette smokers than in nonsmokers and also demonstrated a correlation between the intensity of smoking and the extent of atherosclerosis *Strong et al* (1969), using a standardized visual assessment of the percentage areas of atherosclerotic lesions in

Received 27 xii 71

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TABLE 1 *Smoking Habits by Age and Mechanism of Death*

	Age						
	20–24	25–34	35–44	45–54	55–64	65–	Total
VIOLENT DEATHS							
Cigarette smokers							
less than 25/day	2	–	3	–	2	2	9
more than 25/day	3	8	12	5	9	1	38
the amount unknown	–	2	2	1	1	–	6
total no of cases	5	10	17	6	12	3	53
Non cigarette smokers							
never smoked				2	4	6	17
ex smokers	–	1	3	2	3	2	11
pipe smokers	1	1	–	–	1	–	3
total no of cases	2	5	4	4	8	8	31
CORONARY DEATHS							
Cigarette smokers							
less than 25/day	–	–	–	–	8	3	11
more than 25/day	–	2	12	17	9	4	44
the amount unknown	–	–	–	4	3	1	8
total no of cases	–	2	12	21	20	8	63
Non-cigarette smokers							
never smoked	–	–	–	2	9	3	14
ex smokers	–	–	–	4	9	1	14
pipe-smokers	–	–		3		–	3
total no of cases	–	–		9	18	4	31

arterial specimens and optical electronic scanning of the extent of calcifications from radiographs, found that atherosclerosis of the coronary arteries and aorta was more severe in cigarette smokers than in nonsmokers and that the severity of atherosclerosis was related to the intensity of smoking However Vitel et al (1968) using a similar visual estimation of atherosclerotic lesions observed no correlation between smoking habits and the severity of coronary atherosclerosis in subjects who had died by violence

This report is concerned with the relationship between cigarette smoking and atherosclerosis of the coronary arteries and aorta in two autopsy series, one consisting of men who had died by violence and another of men who had died suddenly from coronary heart disease Point counting technique, a quantitative method for surface area measurements, was employed in the assessment of the extent of atherosclerosis

MATERIAL AND METHODS

The material of the present study consisted of 178 men autopsied at the Institute of Forensic Medicine, University of Helsinki Among these, 84 had died by violence while 94 had died suddenly from coronary heart disease These 178 cases form part of a larger series of 377 men—236 men who had died by violence and 141 men who had died from coronary heart disease—in whom atherosclerosis in the coronary arteries and aorta was studied by quantitative methods In the 178 cases on which this study is based sufficiently reliable information could be obtained concerning smoking habits The median age in the series of violent deaths was 43 years, the range being from 20–77 The median age in the series of coronary disease was 56 years ranging from 30 78 The men comprised in the series coronary heart death had all died within 24 hours after onset of the fatal attack Recent myocardial infarct was observed at autopsy in 46 of these cases (49 per cent) and old infarct scars in 32 (34 per cent) In 16 cases (17 per cent) without any signs of recent or old infarct the diagnosis of coronary death was made on the basis of a previous history of chest pain and/or by previous clinical evidence of the presence of coronary heart

disease and finally by excluding other causes of death

Smoking Habits

Information concerning smoking habits was obtained with the aid of questionnaires from the person who was the next of kin of the deceased. Both series were divided into two categories, cigarette smokers and nonsmokers. The criterion for a cigarette smoker was that he had smoked cigarettes regularly until the time of death. A considerable proportion of the smokers had smoked 25 or more cigarettes per day (Table 1). The "nonsmokers" had never smoked or had stopped smoking at least one year before they died or they had smoked pipes or cigars.

Other Characteristics

The number of cigarette smokers who had been engaged in physically demanding occupations was *higher than that of nonsmokers, a feature seen* particularly in the group of violent deaths. In order to assess the degree of obesity, the mean values of and standard deviations from the ratio of body weight in kg to the square of height in cm × 10 000 (*Khosla & Lowe* 1967) were calculated in the various age groups of smokers and nonsmokers separately in the two series of violent death and coronary death. The values for the smokers were somewhat lower in nearly all groups in both series.

Evaluation of Atherosclerosis

At autopsy the coronary arteries were opened longitudinally as distally as possible. The opened portions of the right coronary artery, the left anterior descending coronary artery and the left circumflex coronary artery were dissected out. The abdominal aorta was longitudinally opened and removed. All specimens were flattened on cardboard and fixed in 10 per cent formalin. The fixed specimens were stained in a Sudan IV solution and radiographs from arterial specimens were taken using a technique described earlier (*Rissanen & Pyörälä*, in press).

The extent of so-called raised lesions as defined in the International Atherosclerosis Project (*Guzman et al.* 1968), i.e. firm elevated intimal lesions with or without other changes covering them or underlying them, was considered to represent the total extent of atherosclerosis in the arterial specimens. In addition, the extent of calcifications was measured from the radiographs of the arterial specimens. The surface areas of raised lesions and calcifications were assessed using the so-called point counting technique. This technique is based on the principle that the number of points overlying the outlines will be proportional to their areas if a large number of points arranged in a given pattern are superimposed over irregular outlines on a plane surface. When irregular outlines of surfaces are measured with the aid of a regular arrangement of points, the absolute surface areas can be calculated. Point counting measurement was performed from aortic specimens by the original method of *Mitchell & Cranston* (1965) and from the coronary artery specimens by a modification of this method (*Rissanen & Pyörälä*, in press). The areas of raised lesions were separately measured in the right coronary artery, the left anterior descending coronary artery and the left circumflex coronary. Similarly, the areas of calcifications were measured from the radiographs of each coronary artery specimen. The areas of raised lesions and calcifications are expressed as absolute values in square centimeters. The results for the three coronary arteries were combined. In addition, the extent of raised lesions is given in per cent of the total wall surface of the coronary arteries and the abdominal aorta. Point counting measurement of the extent of raised lesions as well as calcifications proved to be well reproducible both in the coronary arteries and the aorta; the accuracy was comparable with the accuracy of planimetric measurement (*Rissanen & Pyörälä*, in press).

The degree of obstruction in the coronary arteries was evaluated using the following score:

0 = no stenosis
1 = under 50 per cent stenosis only
2 = one over 50 per cent stenosis but no occlusion
3 = more than one over 50 per cent stenosis but no occlusion
4 = one almost total or total occlusion
5 = several almost total or total occlusions or occlusion extending over several centimeters

The score figures for the right coronary artery, the left anterior descending coronary artery and the left circumflex coronary artery were summed up to a score expressing obstruction of the whole coronary arterial tree. The reproducibility of the assessment of the score as performed by one and the same person proved to be fairly good (*Rissanen* 1970).

All measurements and evaluations were performed by the same person who did not know about the data on smoking habits.

Analysis of the Results

The mean values and standard deviations denoting the extent of raised lesions and calcifications, denoting the scores for obstruction of the coronary arteries and the total surface area of the coronary artery and aortic specimens were calculated for 10 year age groups of cigarette smokers and nonsmokers separately in the series of violent deaths and coronary deaths. The statistical significance of the difference between smokers and nonsmokers in

TABLE 2 *The Mean Values and Standard Deviations for the Absolute Area of Raised Lesions (sq cm) in the Coronary Arteries in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non-cigarette smokers			Cigarette smokers			Non-cigarette smokers		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
20–24	5	0 13	0 26	2	0	0						
25–34	10	1 82	1 60	5	0 64	0 45	2	5 98	4 94			
35–44	17	3 20	1 94	4	0 72	0 42	12	10 8	4 87			
45–54	6	7 25	3 13	4	2 96	2 61	21	9 74	4 14	9	7 45	4 39
55 64	12	6 54	4 46	8	9 38	4 39	20	10 6	3 35	18	12 0	4 36
65 or over	3	8 95	3 90	8	7 34	4 92	8	12 9	2 75	4	9 82	3 94

TABLE 3 *The Mean Values and Standard Deviations for the Percentage Area of Raised Lesions in the Coronary Arteries in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non cigarette smokers			Cigarette smokers			Non-cigarette smokers		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
20–24	5	0 7	1 4	2	0	0						
25–34	10	9 1	9 4	5	3 0	1 8	2	28 9	23 1			
35–44	17	13 8	7 1	4	2 8	1 3	12	42 8	16 1			
45–54	6	35 2	15 3	4	9 6	8 0	21	43 7	16 0	9	33 9	18[illegible]
55–64	12	31 6	21 2	8	42 4	17 6	20	49 8	18 0	18	47 6	16[illegible]
65 [illegible]	3	44 4	16 4	8	25 8	19 2	8	50 0	11 8	4	45 6	6[illegible]

the variables used as estimates of atherosclerosis was tested as follows The unilateral t test was applied to each age group The probabilities of these mutually independent tests were combined using the formula

$$Z = -\sum_{i=1}^{I} 2\ln(1\ P(t_i))$$

where I is the number of tests, t_i the t value of Student s test in age group i and $P(t_i)$ the corresponding p value Z has a χ^2 distribution with 2 × I degrees of freedom (*Pearson* 1938, *Fisher* 1944) In order to obtain the accurate p value for the test for each age group these values were calculated from the t functions with relevant degrees of freedom the p values for Z were obtained from χ^2 tables

RESULTS

Atherosclerosis of the Coronary Arteries

The relative extent of the atherosclerotic lesions being calculated on the basis of total surface area of the coronary artery specimens, it is important to establish that no consistent difference in the latter parameter in cigarette smokers and nonsmokers was observed in

TABLE 4 *The Mean Values and Standard Deviations for the Absolute Areas of Calcifications (sq cm) in the Coronary Arteries in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non-cigarette smokers			Cigarette smokers			Non cigarette smokers		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
20–24	5	0	0	2	0	0						
25–34	10	0	0	5	0	0	2	0	0			
35–44	17	0.07	0.05	4	0	0	12	0.53	0.37			
45–54	6	0.52	0.47	4	0.17	0.12	21	1.12	0.75	9	0.42	0.28
55–64	12	0.86	0.44	8	1.75	1.36	20	1.67	1.57	18	1.51	0.96
65 or over	3	1.15	0.87	8	0.74	0.71	8	1.39	0.81	4	1.74	0.95

TABLE 5 *The Mean Values and Standard Deviations for the Obstruction Score in the Coronary Arteries in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non cigarette smokers			Cigarette smokers			Non cigarette smoker		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
)-24	5	0.2	0.4	2	0	0						
-34	10	1.4	0.9	5	0.6	0.5	2	3.0	1.0			
44	17	2.3	1.0	4	1.0	0.0	12	5.8	2.7			
54	6	4.5	2.6	4	1.5	1.1	21	6.8	3.2	9	5.8	3.7
64	12	5.3	3.5	8	6.5	3.0	20	8.6	3.1	18	8.2	2.5
ver	3	6.3	2.6	8	4.4	3.2	8	8.0	2.1	4	8.0	1.2

TABLE 6 *Significance of Differences between Smokers and Non-Smokers in the Total Area of Coronary Arteries in the Area of Raised Lesions and Calcifications in the Coronary Arteries and in the Coronary Artery Obstruction Score in the Series of Violent Deaths and Coronary Deaths*

riable	Violent deaths			Coronary deaths		
	Z	df	< p <	Z	df	< p <
al area of coronary rteries cm²	9.2	12	.60 < p < .70	5.2	6	.50 < p < .60
ed lesion cm	26.8	12	.005 < p < .01	10.8	6	.05 < p < .10
ed lesion %	33.5	12	< p < .0005	10.0	6	.10 < p < .20
ifications cm	17.9	8	.01 < p < .025	12.9	6	.025 < p < .05
:	28.7	12	.001 < p < .01	6.5	6	.30 < p < .40

TABLE 7 *The Mean Values and Standard Deviations for the Absolute Area of Raised Lesions (sq cm) in the Abdominal Aorta in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non-cigarette smokers			Cigarette smokers			Non-cigarette smokers		
	N	mean	s d	N	mean	s. d	N	mean	s d.	N	mean	s.d.
20–24	5	0.28	0.42	2	0	0						
25–34	10	3.04	5.16	5	1.26	1.61	2	11.9	7.69			
35–44	17	10.0	9.71	4	1.33	0.56	12	17.6	14.7			
45–54	6	21.4	15.8	4	15.9	15.5	21	35.5	14.5	9	26.6	19[illegible]
55–64	12	29.0	11.7	8	24.3	18.5	20	46.2	12.8	18	43.5	24[illegible]
65 or over	3	56.2	4.47	8	33.1	17.7	8	63.2	24.0	4	45.0	9.1[illegible]

TABLE 8 *The Mean Values and Standard Deviations for the Percentage Area of Raised Lesions in the Abdominal Aorta in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non-cigarette smokers			Cigarette smokers			Non-cigarette smokers		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
20–24	5	0.6	0.9	2	0	0						
25–34	10	6.6	10.9	5	2.4	3.0	2	25.3	15.9			
35–44	17	19.6	18.5	4	2.6	1.1	12	31.2	23.5			
45–54	6	35.5	24.6	4	22.9	20.5	21	58.3	19.9	9	45.0	31[illegible]
55–64	12	48.7	18.8	8	33.7	21.0	20	69.3	15.0	18	57.8	2[illegible]
65 or over	3	77.0	12.0	8	46.8	22.9	8	77.8	13.8	4	65.4	14.[illegible]

either series The mean values for the absolute and the relative areas of raised lesions were greater for the cigarette smokers than for the nonsmokers in the series of violent deaths (Tables 2 and 3), except in the age groups 55–64 years In this series calcifications were found in the four oldest age groups With the exception of the age group 55–64 years, the mean area of calcifications was greater in smokers than in nonsmokers in the series of violent deaths (Table 4) In this series, the mean values for the obstruction score were also higher in smokers than in nonsmokers, again with the exception of the age group 55–64 years (Table 5) Tested as described in the foregoing, the difference between smokers and nonsmokers was significant for all these variables (Table 6)

In the series of coronary deaths only three age groups over 45 were available for a comparison of smokers and nonsmokers The mean values for the variables studied are, however also given for the age groups under 45, consisting exclusively of smokers to enable comparison with the data on men who had died by violence and with the data on the older age groups in the series of coronary death In the three age groups in the series of

TABLE 9 *The Mean Values and Standard Deviations for the Absolute Area of Calcifications (sq cm) in the Abdominal Aorta in Cigarette Smokers and in Non Cigarette Smokers in the Series of Violent Deaths and Coronary Deaths, by Age*

Age	Violent deaths						Coronary deaths					
	Cigarette smokers			Non cigarette smokers			Cigarette smokers			Non-cigarette smokers		
	N	mean	s d	N	mean	s d	N	mean	s d	N	mean	s d
20 24	5	0	0	2	0	0						
25 34	10	0 03	0 09	5	0	0	2	0 17	0 17			
35–44	17	0 35	0 53	4	0	0	12	1 60	2 54			
45 54	6	4 26	8 40	4	2 34	3 67	21	4 79	3 94	9	3 04	4 23
55–64	12	5 59	7 30	8	5 38	4 02	20	8 73	7 05	18	6 04	6 18
65 or over	3	15 5	9 47	8	6 64	7 91	8	8 86	4 95	4	6 64	4 09

TABLE 10 *Significance of Differences between Smokers and Non Smokers in the Total Area of Abdominal Aorta and in the Area of Raised Lesions and Calcifications in the Abdominal Aorta in the Series of Violent Deaths and Coronary Deaths*

Variable	Violent deaths			Coronary deaths		
	Z	df	< p <	Z	df	< p <
Total area of abdominal aorta cm²	10 7	12	50 < p < 60	7 7	6	20 < p < 30
Raised lesion cm	24 5	12	01 < p < 025	11 8	6	05 < p < 10
Raised lesion %	25 8	12	01 < p < 025	15 9	6	01 < p < 025
Calcifications cm	15 7	10	,10 < p < 20	11 2	6	05 < p < 10

coronary death available for comparison, there was no consistent difference between smokers and nonsmokers in the absolute and relative area of raised lesions (Table 2 and 3) In the age group 45–54 years the mean absolute area of calcifications was markedly greater in smokers than in nonsmokers and a slight trend to the same direction was observed in the age group 55–64 years (Table 4) The coronary artery obstruction score did not show any remarkable differences between smokers and nonsmokers (Table 5) The only difference between smokers and nonsmokers in the series of coronary deaths reaching the level of statistical significance was that found in the absolute area of calcifications (Table 6)

Atherosclerosis of the Abdominal Aorta

There was no consistent difference between smokers and nonsmokers in the two series as regards the total surface area of the abdominal aorta Both the absolute and the relative area of raised lesions was greater in smokers in all age groups than in nonsmokers in the series of violent death (Tables 7 and 8) The same was true for calcifications in all age groups in which such lesions were observed (Table 9) The difference was statistically significant for both the absolute and the relative area of raised lesions, but the difference in the absolute area of calcifications did not attain the level of statistical significance (Table 10)

In the three oldest age groups in the series of coronary deaths the absolute and the relative area of raised lesions and the absolute area of calcifications were greater in smokers than in nonsmokers (Tables 7, 8, 9), but the difference was statistically significant only for the relative area of raised lesions (Table 10)

Occupational Physical Activity in Relation to Coronary and Aortic Atherosclerosis

The smokers and nonsmokers differed somewhat with regard to distribution into various groups of occupational physical activity In order to analyse the possible effect of this factor on the results the mean values and standard deviations for the absolute area of raised lesions in the coronary arteries and abdominal aorta were calculated for men representing different levels of occupational physical activity in the various age groups both in the series of violent deaths and in the series of coronary deaths The number of cases in the two series was too small to allow this analysis to be made separately in the series of cigarette smokers an nonsmokers No obvious correlation between the area of raised lesions in the coronary arteries and aorta and the level of occupational physical activity was observed

DISCUSSION

Close examination of atherosclerosis in the arterial walls is only feasible post mortem In general, however, an autopsy series does not represent, unbiased the corresponding living population (*Mainland* 1953 *McMahan* 1962 *Sternby* 1968) When atherosclerosis is the object of study, an autopsy series consisting of subjects who have died by violence may be considered to represent a sample most closely approaching a cross-section of the general population Among previous investigations in which atherosclerosis of the coronary arteries or aorta is correlated to smoking habits only that by *Viel et al* (1968) was performed on subjects who had died by violence The series reported by other investigators consisted exclusively of hospital cases (*Auerbach et al* 1965 *Sackett et al* 1968) or included subjects who died by violence and subjects who died from diseases not related to atherosclerosis (*Strong et al* 1969)

In medico-legal autopsy cases, reliable information about smoking habits is not obtained as easily as in autopsy cases from hospitals Hence such information was obtained only in 47 per cent of the cases in the series of medico legal autopsies on which the present study was based In spite of this, however, the material of this study may be considered suitable for the investigation of the relationship between smoking and atherosclerosis since, in regard to age distribution occupation and body constitution in the two groups violent deaths and coronary deaths findings in men whose smoking habits were not known did not differ significantly from those in men whose smoking habits were well-established

In the present autopsy series of men who had died by violence atherosclerosis in the coronary arteries and aorta was consistently more severe in regular cigarette smokers than in nonsmokers In the present study persons who had stopped cigarette smoking and pipe or cigar smokers were included into the group of nonsmokers since the size of the series did not allow a separate analysis of all these smoking categories No matter the type of linkage between cigarette smoking and atherosclerosis, the correlation found in the present study between cigarette smoking and the severity of atherosclerosis evidently was to some extent underestimated since ex smokers may be closer to current smokers with regard to atherosclerotic involvement The results of the present study are in agreement with the results of some previous investigations from U S A (*Wilens* & *Plair* 1962 *Auerbach et al* 1965, *Sackett et al* 1968 *Strong et al* 1969) but differ from the observations reported by *Viel et al* (1968) from Chile who found no relationship between smoking and coronary atherosclerosis in a series of violent deaths However, the series of *Viel et al* was collected from a population with a low

prevalence of atherosclerosis (*Tejada et al* 1968)

The other series of the present study comprising men who had died from coronary heart disease soon after the onset of symptoms of the fatal attack is a selected group of subjects with this disease Persons to be included into the group of sudden coronary deaths generally show an extensive involvement of the coronary arteries by atherosclerosis Hence, it could be expected that, in a series selected according to this mode of death, smoking would not show any relationship to the severity of coronary atherosclerosis The results of the present study conformed to that expectation However, also in the series of coronary deaths, aortic atherosclerosis tended to be more severe in smokers than in nonsmokers

In the present study it was not possible to analyse the relationship of the intensity of smoking to the coronary and aortic atherosclerosis because of the small size of the series However, other investigators have found in larger series that both the amount of cigarettes smoked per day and the duration of smoking show a relationship to the severity of coronary atherosclerosis (*Auerbach et al* 1965, *Strong et al* 1969) and aortic atherosclerosis (*Sackett et al* 1968, *Strong et al* 1969) These findings favour the view that cigarette smoking *per se* may by some mechanism enhance the development of atherosclerosis rather than the view that constitutional and genetic factors related to cigarette smoking also are related to increased susceptibility to atherosclerosis

It has been found in several studies that the degree of obesity is not related to the severity of coronary and aortic atherosclerosis (*Giertsen* 1966, *Montenegro & Solberg* 1968, *Sternby* 1968, *Rissanen & Pyörälä*, unpublished results), As generally seen, cigarette smokers in the present series were *thinner* than nonsmokers In the series of violent deaths in the present study the proportion of men whose level of occupational physical activity was high was greater in the group of cigarette smokers than in that of nonsmokers However, in accordance with *Strong et al* (1969), the level of occupational physical activity did not show any relationship to the severity of coronary and aortic atherosclerosis Heavy labour is generally associated with a low social class Hence, smokers and nonsmokers in the present series may have differed with regard to factors such as diet, stress and life habits associated with socioeconomic levels However, evaluation of the possible relation of these and other confounding variables to the severity of coronary and aortic atherosclerosis was not possible in the present study and for obvious reasons it would be difficult even in studies based on more extensive autopsy series

This study was supported by grants from *Paavo Ilmari Ahvenainen Foundation*, from *The Finnish Heart Association* and from *The National Research Council for Medical Sciences in Finland*

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Acta path microbiol scand Section A, 80, 501–508, 1972

ENZYME STUDIES IN MAN WITH EXTRA-HEPATIC BILIARY OBSTRUCTION

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In an investigation of liver biopsy specimens from patients with extra hepatic biliary obstruction an increase was found in the products of histochemical reactions for acid phosphatase, beta-glucuronidase non specific esterase and succinic dehydrogenase around bile pigments, for alkaline phosphatase in sinusoids and canaliculi, for adenosine triphosphatase in sinusoids and some canaliculi with loss in others Leucine aminopeptidase outlined irregular and thickened canaliculi, gamma glutamyl transpeptidase increased number of stained thin canaliculi

The histochemical behaviour of a few enzymes in the human livers has been the the subject of some relatively recent publications (4 7, 8 9, 11) This paper reports a fairly comprehensive study of 11 enzymes representing different cell organelles and especially enzymes known to be increased in the serum during biliary obstruction

MATERIAL AND METHODS

Liver biopsy specimens (about 1×1×2 cm) were obtained at operation on 28 patients with obstruction of the common bile duct (pathological material) and from 5 patients operated upon because of peptic ulcer (control material) The material is surveyed in Table 1 All the patients suffering from biliary obstruction were anaesthetized with pentothal sodium nitrous oxide-oxygen and phenoperidin® the controls, with pentothal sodium nitrous oxide-oxygen and halothane® The specimens were kept at 0° C for 10 to 20 minutes after which they were frozen in carbon dioxide snow and stored at —70° C for some days

Cryostat sections were studied for enzyme activities (succinic dehydrogenase, non-specific esterase, monoamine oxidase acid and alkaline phosphatase, adenosine triphosphatase, gamma glutamyl trans peptidase) with the techniques described elsewhere (2) In addition in some of the cases sections were also stained for leucine aminopeptidase beta glucuronidase glucose 6-phosphatase and lactic dehydrogenase Staining for leucine aminopeptidase was performed according to *Nachlas et al* (1960), for beta glucuronidase with the method of *Hayashi et al* (1964) for glucose-6 phosphatase according to *Wachstein & Meisel* (1956) for lactic dehydrogenase, in the way described by *Barka & Anderson* (1963 p 314) but tetra Nitro-BT was used instead of Nitro-BT and to the final incubation solution was added polyvinyl pyrrolidone (mol wt 10,000) to a final concentration of 20 per cent after which the pH was adjusted (1)

Control sections without substrates were not regularly studied but sections from a normal rat liver were always used as controls When the specimen was large enough material was also fixed in 10 per cent formalin, frozen and stained for fat (Sudan black or Scharlach rot) and paraffin

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embedded and stained with haematoxylin erythrocin and periodic acid Schiff (PAS) according to Mac Manus (with and without diastase digestion) Values for serum bilirubin, glutamate pyruvate transaminase, alkaline phosphatase and gamma glutamyl transpeptidase were available from the case records (for methods, see (2))

RESULTS

Control Material

Histology The five biopsy specimens were of normal appearance

Histochemistry Sections stained for *succinic dehydrogenase and monoamine oxidase* activities contained fine and coarse granules of formazan throughout the hepatic plates Staining for monoamine oxidase activity sometimes revealed coarse granules situated along sinusoids (also with tetra-Nitro-BT)

Non specific esterase As a rule, the red reaction product was evenly distributed throughout the hepatic plates, somewhat stronger in the centrolobular halves

Acid phosphatase and beta-glucuronidase The sections showed a faint positive reaction in pericanalicular lysosomes and a strong diffuse reaction of acid phosphatase in Kupffer cells

Alkaline phosphatase activity was found in the wall of central veins and surrounding sinusoids as well as in periportal arteries, veins and sinusoids adjacent to the limiting plate No staining of biliary passages was seen

Adenosine triphosphatase The sections showed a general canalicular as well as sinusoidal pattern and positive vessels in portal zones The plasma membrane of the biliary epithelium showed a faint reaction somewhat stronger apically

Gamma glutamyl transpeptidase activity was demonstrated in the epithelium of bile ducts and ductules and in periportal canaliculi

Leucine aminopeptidase activity revealed thicker and more knotty canaliculi than sections stained for adenosine triphosphatase activity Also the sinusoidal walls were positive stronger in the periphery of the lobules Vessels and biliary ducts in the portal zones stained

Lactic dehydrogenase The sections showed evenly distributed cytoplasmic fine formazan granules throughout hepatic plates

Glucose-6-phosphatase representing cyto membranes showed even cytoplasmic staining stronger periportally than centrolobularly

Pathological Material

Histology All the biopsy specimens showed evidence of cholestasis with bile casts in canaliculi and bile pigment in hepatocytes and Kupffer cells These changes were always found in the central parts of the lobules and in advanced cases also peripherally Only one case (T 6161/68) showed bile lakes and bile stained material in biliary ducts and ductules Except for cholestasis the morphological

TABLE 1 *Survey of the Material*

Biopsy and autopsy no	Diagnoses	Sex	Age	Serum studies on same day as biopsy				Duration of jaundice before biopsy Days
				GPT	Bil	Alk	GT	
CONTROL MATERIAL								
T 8046/70	ulcus duodeni	m	39	11	0 39	5	49	
T 8424/70	ulcus duodeni	m	36	16	0 8	4	36	
T 1086/70	gastritis	w	84	25	0 37	9	140	
T 3176/70	ulcus ventriculi	m	51		1 19			
T 5134/70	ulcus duodeni	m	57	–	0 74		–	

TABLE 1 *Continued*

Biopsy and autopsy no	Diagnoses	Sex	Age	Serum studies on same day as biopsy GPT	BiA	AIk	GT	Duration of jaundice before biopsy Days
PATHOLOGICAL MATERIAL								
T 6933/67 O 169/68	cancer vesicae felleae	m	57	60	–	27	1740	35–40
T 9526/67	cholecysto and choledocholithiasis	m	56	100	7.9	28	1245	14
T 9527/67 O 243/68	cancer caput pancreatis	w	72	55	13.1	53	2050	18
T 16703/67 O 1706/67	haemangioma cavernosum caput pancreatis	w	73	162	33	43	2570	21
T 18351/67 O 76/69	cancer ductuum hepatici	m	75	630	16	49	3800	30
T 1100/68	cholecysto- and choledocholithiasis	w	45	324	9.8	39	598	14–18
T 1501/68	cancer pancreatis	m	51	26	27	54	830	30
T 4800/68 O 1035/68	cancer pancreatis	m	52	96	40.7	29	1258	30
T 5104/68 O 880/68	cancer pancreatis	w	68	156	14	72	5020	40
T 6161/68 O 1213/68	cancer choledochi	m	71	95	30	23	1884	30
T 16696/68	cholecysto- and choledocholithiasis	w	24	285	6.4	11	130	16
T 547/69	cholecysto- and choledocholithiasis	w	64	56	17.2	26	435	60
T 614/69 S 345/69	cancer vesicae felleae	w	69	85	31	15	355	30
T 2747/69	cholecysto- and choledocholithiasis	w	70	77	1.1	16	355	35
T 2748/69	cholecysto- and choledocholithiasis	w	82	115	1.0	88	3710	24
T 2908/69 O 506/69	cancer vesicae felleae	w	41	260	11.4	41	1210	28
T 3738/69	status post lesio choledochi	w	59	86	15.0	54	342	4 years
T 4969/69	cancer pancreatis	m	70	122	11.9	32	895	21
T 5512/69	cholecystolithiasis	w	45	260	2.2	10	270	16
T 5602/69	cholecysto- and choledocholithiasis	m	83	65	5.9	36	560	40
T 6861/69	cholecysto- and choledocholithiasis	w	82	825	1.0	24	135	7
T 6884/69 O 623/69	cancer pancreatis	m	65	75	8	26	1060	21
T 8299/69	cholecysto- and choledocholithiasis	w	24	225	11.3	10	165	18
T 11273/69	cholecystolithiasis	w	59	41	0.69	5	35	25
T 13516/69	cholecystolithiasis	w	48	89	12.4	21	47	34
T 14056/69	cholecysto- and choledocholithiasis	w	22	81	7.58	35	335	14
T 15731/69	cancer vesicae felleae	m	63	51	17.5	57	1455	35
T 16653/69	cholecysto- and choledocholithiasis	m	30	118	4.3	34	418	18

appearance was sometimes normal but most often there were changes in portal zones and around central veins From portal zones connective tissue strands grew out through the limiting plate and contained increased numbers of ductules and ducts as well as inflammatory cells (neutrophil leucocytes and lymphocytes) Centrolobularly thin fibrous strands and inflammatory cells were seen besides hepatocytes with necrosis as well as fatty degeneration and cloudy swelling In one case (T 4800/68) the cytoplasm of the centrolobular hepatocytes contained irregular homogeneous areas with diastase resistant PAS positive material In 3 cases (T 16703/67 T 1501/68 T 6156/68) the cytoplasm of groups of hepatocytes had lost its eosinophilia and the cells had rounded contours The Kupffer cells often seemed to be increased in number especially centrolobularly where their cytoplasm contained diastase resistant PAS positive material (Fig 1)

Histochemistry Succinic dehydrogenase Fine as well as coarse granules of formazan were seen throughout the hepatic plates Centrolobular necrotic cells contained no formazan granules but other cells showed irregularly distributed coarse granules surrounded by empty halos In 9 cases some hepatocytes situated around bile casts showed a higher concentration of finely granular formazan than did surrounding cells The groups of degenerating hepatocytes seen in 3 cases showed faint formazan deposits In some cases staining of the entire specimen was very weak in spite of a normal intensity of the stain in the control rat liver (Fig 2)

Fig 1 T 6161/68 Centrolobular region The dark biliary lump in the centre is surrounded by a zone of non glycogenic PAS positive material Swollen pale hepatocytes and some PAS positive Kupffer cells are seen Mac Manus after diastase digestion × 680

Fig 2 T 6861/69 Stronger reaction for succinic dehydrogenase activity in cells around the bile deposits Upper left × 360

Fig 3 T 5602/69 Monoamine oxidase with coarse formazan reaction product along sinusoid × 115

Monoamine oxidase activity showed the same picture as for succinic dehydrogenase with unstained necrotic hepatocytes centrolobularly but never increased staining in hepatocytes adjacent to bile cast or bile pigment Sometimes coarse formazan granules were demonstrated along sinusoids The groups of degenerating hepatocytes observed in 3 cases stained only weakly (Fig 3)

Non specific esterase As a rule, the red reaction product was evenly distributed throughout the hepatic plates but centrolobular necrotic hepatocytes were unstained In six cases the staining was stronger centrolobularly within and around areas with bile pigment The groups of degenerating hepatocytes observed in 3 cases did not stain

Acid phosphatase Red azo dye indicating enzyme activity was invariably accumulated in regions with bile pigment in the form of large lumps partly or totally enclosing bile material although bile pigment without such lumps was also seen (Fig 4) Only in a few cases could the lumps of azo-dye be seen as aggregates of dots Outside areas with biliary pigment there was more intense lysosomal pattern in hepatocytes and Kupffer cells The groups of degenerating hepatocytes seen in 3 cases were stained diffusely red by the acid phosphatase methods (Fig 8)

Beta glucuronidase was estimated in 18 cases Numerous lumps with granular positive material were seen around bile material and inside hepatocytes a pericanalicular reaction radiating outwards from areas of bile stasis in the lobules

The alkaline phosphatase reaction was very strong with enzyme product along sinusoids and with a marked increase in central parts of the lobules The sinusoids stained intensely



Fig 4 T 6161/68 Acid phosphatase reaction with dark lumps of biliary pigment and azo-dye product centrolobularly × 184

Fig 5 T 547/69 Alkaline phosphatase with strong sinusoidal and canalicular reaction × 300

Fig 6 T 8424/70 Adenosine triphosphatase Normal liver × 300

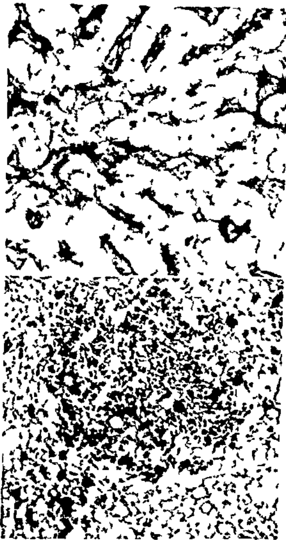


Fig 7 T 6861/69 Adenosine triphosphatase g sinusoidal activity Loss of canalicular pat × 300

Fig 8 T 16703/67 A group of degenerating hepatocytes with a strong acid phosphatase reaction (other enzyme products were decreased) × 75

around the groups of degenerating hepatocytes observed in 3 cases In 8 cases discontinuous canalicular deposits of reaction products were seen (Fig 5) In the portal connective tissue the wall of the portal vein and the intima of the hepatic artery were stained while bile ducts and ductules were constantly negative

Adenosine triphosphatase Sometimes both canalicular and sinusoidal structures were positive but more often the canaliculi did not stain and the walls of the central sinusoids were heavily stained (Fig 6 7) In some cases the entire plasma membrane around the hepatocytes was positive In the portal zones a reaction product was seen in the portal vein and the hepatic artery as well as in bile ducts and ductules

The gamma glutamyl transpeptidase reaction showed a precipitation in the epithelium of bile ducts and ductules and mostly in periportal canaliculi In 10 cases thin canaliculi were depicted throughout the lobules In a few cases a barely discernible reaction was seen along sinusoids

Leucine aminopeptidase (assayed in 24 cases) Irregular knotty canalicular structures were seen with a widening around bile casts surrounded by broad strands of reaction products In some places the reaction was negative around bile casts The epithelium of bile ducts and ductules was positive In most cases the sinusoids stained but in some only faintly

Lactic dehydrogenase In 9 cases studied the picture appeared normal

Glucose-6-phosphatase was estimated in 6 cases The activity was decreased especially in the central two thirds of the lobules

DISCUSSION

The enzyme histochemistry of the cholestatic human liver has received relatively little attention A review of the literature is given in Table 2 *Kecht et al* (1960) published a brief survey of the literature including a report of a personal study of alkaline phosphatase in biliary obstruction They found the azo coupling reaction as well as Gomori s reaction to show a high alkaline phosphatase activity in sinusoids and bile capillaries but no activity in bile duct epithelium *Holzner* (1960) reported an increased sinusoidal activity of adenosine triphosphatase in liver biopsy specimens from patients with mechanical biliary obstruction *Holzner et a* (19 3) found *inter alia* acid phosphatase act

TABLE 2 *Review of Enzyme Histochemical Studies of Human Hepatic Tissue from Cases with Extra-Hepatic Biliary Obstruction*

Authors	Material	Enzyme reactions	Results in summary
Kechk et al 1960	Surgical biopsies	Alkaline phosphatase	Increase in sinusoids and bile capillaries, negative bile duct epithelium
Holzner 1960	Needle biopsies (?)	Adenosine triphosphatase	Increase in sinusoids
Holzner et al 1963	Needle biopsies (?)	Adenosine triphosphatase Acid phosphatase	Increase in sinusoids increase and decrease to total loss in canaliculi Increase in hepatocytes with scattered granules
Wills & Epstein 1966	Surgical biopsies	Adenosine triphosphatase (electron microscopy)	Loss of activity in villi of bile capillaries
Ekelund 1971	Fine needle biopsies (cytology)	Leucine aminopeptidase	Canalicular dilatation Diffusion of the canalicular margin

demonstrated by Gomori s and by azo-coupling methods, to be increased with scattered granules in the cytoplasm of hepatocytes In an electron microscopic study, *Wills & Epstein* (1966) found adenosine triphosphatase activity to be reduced in canaliculi owing to loss of microvilli The observations made in the present study corroborate findings on record Increased acid phosphatase activity also in intrahepatic cholestasis has been reported (11) In the present material such an increase presented partly as pericanalicular lysosomes partly as Kupffer cells and partly as large intracellular homogenous lumps, while the reports referred to above described granular products resembling those found by us in material stained for beta-glucuronidase activity We have been able to confirm the occurrence of these large areas of acid phosphatase activity in material stained according to Gomori and in unfixed and in formalin-fixed as well as in acetone fixed material A more extensive discussion will be given in a separate report where cholestatic human, dog and rat livers will be compared in respect of the histochemical behaviour of their enzymes

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Acta path microbiol scand Section A 80, 509-514, 1972

HISTOLOGICAL TYPING OF GASTRIC CARCINOMA

A Comparison of Surgical and Autopsy Materials, and of Primary Tumours and Metastases

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Histological classification of gastric carcinomas into the main types intestinal and diffuse was made according to *Laurén's* method Autopsy material was compared to surgical material, and metastases were compared to primary tumours, with regard to type distribution and reproducibility The proportion of diffuse tumours was substantially higher in the total autopsy material than in the total surgical material This was probably due to a stronger tendency for diffuse tumours to reach an inoperable stage before the diagnosis is made In patients from whom both surgical and autopsy specimens were available, the same type was diagnosed in both in the majority of cases, and the proportion of diffuse tumours was only slightly higher if typed from the autopsy material than if typed from the surgical material The reproducibility of histological typing in two independent readings of the same sections was 91 per cent in surgical material and 84 per cent in autopsy material Metastases reproduced the histological type of the primary tumour in 65 per cent for all metastatic sites combined Diffuse patterns were most frequently reproduced in metastases to adipose and fibrous tissues and intestinal patterns were most frequently reproduced in metastases to the liver

The histological classification of gastric carcinoma into the two main types intestinal and diffuse, suggested by *Lauren* in 1965, has added a new dimension to the epidemiology of gastric cancer The two histological types have different sex and age distributions (*Lauren* 1965, *Muñoz et al* 1968), they occur in different relative proportions in populations with high and low incidence of gastric cancer (*Muñoz et al* 1968, *Muñoz & Connelly* 1971), they show different time trends (*Muñoz & Connelly* 1971, *Muñoz & Asvall* 1971), and the intestinal type is associated with intestinal metaplasia of the non-tumorous gastric mucosa (*Lauren* 1965, *Muñoz et al* 1968, *Correa et al* 1970)

Such observations have supported the view that the two histological types may be different aetiological entities and encourage the use of histological classification in epidemiological search for risk factors in gastric carcinoma

In the studies published so far, the *Lauren* classification has been applied to surgical material only Some studies have been restricted to gastric resection specimens, others do not specify whether metastatic tumours were included For the comparison of histo

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logical types of gastric carcinoma from different places or time periods, it is essential to know to what extent differences in the sources of material might influence the results

The purpose of the present study has been to examine the validity of histological typing in autopsy material and in metastatic tumours, and the effect of including such data in epidemiological studies

MATERIAL

The study is based upon histological material taken for routine examination at Ullevål Hospital which serves the City of Oslo as a general hospital, the material includes 1 *All surgical specimens from* patients in whom the histological diagnosis of stomach cancer was made for the first time during the three year period 1963-65, 268 patients 2 All autopsies from the same period on patients in whom stomach cancer tissue was present at autopsy and removed for histological examination, 180 *patients* 3 *Surgical specimens from earlier years* from patients included in the autopsy material 18 patients The last set of material was only used in the studies on histological type reproducibility (Tables 5 to 8)

METHOD

Autopsies had been performed 8 32 hours *post mortem* in most cases

Histological typing was made without any information about the patients except specimen numbers The typing was repeated in reverse order and without knowledge of the results of the first reading after an interval of two weeks In cases of disagreement between first and second readings the type was determined in a third review of the slides From specimens represented by multiple slides those showing metastatic tumour only were sorted out thoroughly mixed and typed in a single reading

Typing followed the criteria set up by *Laurén* (1965) and *Muñoz et al* (1968) The pattern of growth and the presence or absence of intestinal type glands with brush border were the main criteria used, little importance was attached to nuclear morphology and mucus secretion

OBSERVATIONS

Among 366 malignant tumours of the stomach diagnosed histologically for the first time during 1963-65, 268 were diagnosed on the basis of surgical specimens and 98 at autopsy The fraction of primary histological diagnoses made at autopsy increased with age and reached 44 per cent in patients of 75 years and older (Table 1) Ten of the tumours

TABLE 1 *The Fraction of New Histological Diagnoses of Gastric Cancer Made at Autopsy*

Age	Total new diagnoses	Diagnosed at autopsy No	%
55	59	6	10
55 64	82	17	21
65 74	120	29	24
75	105	46	44
All ages	366	98	27

TABLE 2 *Ratio of Intestinal to Diffuse Carcinomas by Sex Age, and Source of Material Actual Numbers of Cases (Intestinal + Diffuse) in Brackets*

	Age 54	55-64	65 74	75
Men				
Autopsy material	0 9 (13)	0 7 (19)	0 8 (31)	1 9 (26)
Surgical material	0 9 (26)	2 0 (33)	2 7 (48)	2 8 (30)
Surgical excluding metastases	0 6 (18)	2 1 (25)	3 3 (39)	3 0 (16)
All new histological diagnoses	0 9 (30)	1 6 (41)	2 1 (65	2 7 (48)
Women				
Autopsy material	0 2 (6)	0 5 (17)	0 8 (16)	1 1 (23)
Surgical material	0 3 (19)	0 8 (22)	2 8 (23)	1 9 (20)
Surgical excluding metastases	0 2 (15)	0 8 (16)	2 4 (17)	1 8 (14)
All new histological diagnoses	0 2 (21)	0 7 (30)	2 3 (30)	1 5 (38)

TABLE 3 *The Fraction of Carcinomas of Types Other than Intestinal or Diffuse*

	Men	Women
Autopsy material	18 %	14 %
Surgical material	12 %	19 %
Surgical excluding metastases	12 %	18 %
All new histological diagnoses	13 %	17 %

TABLE 4 *Histological Types According to Site of Tumour Surgical Material*

	I	D	Other	I/D
Canalis	59	38	17	1 6
Corpus	40	21	9	1 9
Cardia	3	2	2	1 5
Unspecified	33	25	11	1 3

I intestinal type D diffuse type

TABLE 5 *Reproducibility of Histological Typing in Two Independent Readings of the Same Sections*

	Same type	Different types
Surgical material	259 (91 %)	27 (9 %)
Autopsy material	152 (84 %)	28 (16 %)

(2 7 per cent) were sarcomas The following observations refer to the carcinomas only

The distribution of histological types has been estimated by the intestinal to diffuse (I/D) ratio and by the relative size of the group of other types In Table 2 the autopsy material includes all autopsied cases with intestinal or diffuse type as determined from tissues obtained at autopsy Surgical material is shown both as including and as excluding patients from whom only metastatic tumour tissue was available in surgical material When both primary and metastatic tumour tissues were available preference was given to the type of the primary tumour The group of all new histological diagnoses comprises the surgical material plus cases diagnosed histologically for the first time at autopsy

For all sets of material there was a clear tendency for the I/D ratio to increase with age and to be higher in men than in women Except in the youngest age group, the autopsy material showed substantially lower I/D ratios than any of the other sets of material The exclusion of metastases from the surgical material had no systematic effects on the ratios The ratios of the all new diagnoses material were intermediate between those of autopsy and surgical materials The fraction of carcinomas that were classified as being neither intestinal nor diffuse was essentially the same in all sets of material (Table 3)

The distribution of histological types according to site of the primary tumour is given in Table 4 The I/D ratio was slightly lower in the canalis than in the corpus and was still lower in the tumours of unspecified site

The reproducibility of histological typing in two independent readings of the same sections was 91 per cent in the surgical material and 84 per cent in the autopsy material (Table 5) In each set of material typing was made from the primary tumour when present and from metastatic tumour tissue when this was the only material available These reproducibility rates should be kept in mind when the reproducibility of types is evaluated in histological materials from different sources in one and the same patient

In 76 patients sections from tumour tissues from both autopsy and surgical materials were available for review In Table 6 the tumours that were referred to the other group because of the presence of substantial amounts of both intestinal and diffuse growth patterns (*Muñoz et al* 1968) have been listed separately as I+D Diagnostic changes between this combined type and either of the two main types occurred in both directions and were responsible for about half of the disagreements between surgical and autopsy readings The reproducibility rate of 62 per cent involves both the variation between independent readings and the variation between different specimens It is substantially higher than the random reproducibility rate of 34 per cent to be calculated from the distribution of types in the totals As seen from the totals there was only a slight rela

TABLE 6 *Histological Types in Surgical and Autopsy Material from the same Patients*

		Autopsy material I	D	I+D	Other	Total surgical
Surgical material	I	*19*	3	3	3	28
	D	1	*25*	4	2	32
	I+D	4	4	*1*	–	9
	Other	2	3	–	*2*	7
	Total autopsy	26	35	8	7	76

Same type 47 (62 %)
Different types 29 (38 %)

tive increase of diffuse types from surgical to autopsy material in this set of patients However, even as judged from the surgical specimens, the I/D ratio (0 9) was substantially lower than the average for the total surgical material (1 6) This indicates that among operated patients, tumour tissue is most regularly found at autopsy of those with diffuse tumours Corroborating this suggestion was the observation that among twelve operated stomach cancer patients from the same period in whom no tumour tissue was detected at autopsy, seven had tumours of intestinal type and only two had tumours of diffuse type

A comparison of the histological types of primary and metastatic tumours in the combined autopsy and surgical material is shown in Table 7 Each microscopical slide with sections from one or more pieces of metastatic tumour tissue was typed and counted as one metastasis The rate at which the histological type of the primary tumour was reproduced in the metastases depended both upon the type of the primary tumour and upon the site of the metastasis In adipose and fibrous tissues metastases from diffuse tumours showed diffuse growth patterns with very few exceptions whereas metastases from intestinal type tumours more frequently changed from intestinal to diffuse type In the liver, the reverse situation prevailed No liver metastasis from an intestinal tumour had a diffuse pattern, whereas more than half of the liver metastases from diffuse tumours showed intestinal patterns Lymph node metastases were intermediate in this respect with about equal rates of type change in both *directions* The distribution of metastases from intestinal and diffuse tumours as seen in this routine material is puzzling Liver metastases were most frequently derived from primary tumours of intestinal type, whereas metastases to adipose and fibrous tissues and to the ovary were most frequently derived from tumours of diffuse type In this respect also metastases to lymph nodes showed an intermediate pattern

TABLE 7 *Histological Types in Primary Tumours and their Metastases to Various Secondary Sites Numbers are Independently Read Slides from Metastatic Tumours*

ite of netastasis	Primary I			D			Other			All types	No pat
Metastasis	I	D	Other	I	D	Other	I	D	Other		
Adipose and fibrous tissues	*30*	8	5	*2*	*113*	3	6	*15*	*8*	190	11
ymph nodes	*73*	12	10	12	*60*	21	14	20	*11*	233	14
Liver	*40*		8	12	*3*	5	1	6	2	77	[illegible]
Dvary	*1*	1	–		*10*	–				12	[illegible]
Other	*11*	7	7	11	*26*	10	3	7	2	84	6

TABLE 8 *Per Cent Agreements of Histological Type of Metastasis with Histological Type of Primary Tumour, According to Type of Primary and Site of Metastasis*

Site of metastasis	Type of primary tumour I	D	Other	All types
Adipose and fibrous tissues	70	96	28	79
Lymph nodes	77	65	24	62
Liver	83	15	22	58
Ovary	50	100	–	92
Other	44	55	17	46
All sites	73	74	24	65

The rates of reproducibility of primary histological types in the metastases are summarized in Table 8 When all sites are included, intestinal and diffuse types are reproduced at about equal rates For the individual sites, the rates vary considerably For primary tumours of types other than pure or predominantly intestinal or diffuse, the reproducibility rates are low for all metastatic sites

DISCUSSION

Autopsy material can be typed according to *Lauren's* (1965) classification with a reproducibility not much poorer than typing based upon surgical material The type determined at autopsy was in most cases the same as that determined in surgical material when both were available from the same patient In the cases in which different types were diagnosed from autopsy and surgical materials, much of the disagreement must be ascribed to the fact that the specimens represented different areas of tumour growth, and the difference in quality between surgical and autopsy materials can only be responsible for a minor number of disagreements Thus, as far as validity of histological typing is concerned there should be no objection to the use of autopsy material in comparative studies of the distribution of histological types of gastric carcinoma in different populations As the proportion of diffuse tumours was substantially larger in autopsy material than in surgical material however, no conclusion on population differences should be based upon a comparison between surgical material from one population and autopsy or mixed material from another population

When only metastatic tumour tissue is available, this may be used for typing since most metastases reproduce the histological type of the primary tumour The inclusion of metastases with gastrectomy specimens did not significantly alter the distribution of types in the surgical material Metastases to adipose and fibrous tissues, lymph nodes, and liver should all be included to maintain a balanced representation of types, as the distribution of types was markedly different in the three sites

Surgical practice may influence the distribution of types Because of the different distribution of types in various metastatic sites any systematic change in the practice of selecting the site of biopsy when multiple sites are available will influence the apparent distribution of types Because of the presumably greater tendency of diffuse carcinomas to reach an inoperable state before they are diagnosed, the attitude of the surgeons towards explorative laparotomy in clinically advanced cases, as well as the frequency by which a biopsy is taken from tumours that at laparotomy are found to be inoperable will influence mainly the number of diffuse tumours that are diagnosed histologically The slightly higher proportion of intestinal tumours in the upper parts of the stomach than in the canalis which can also be seen from the data of *Lauren* (1965), may influence type distribution in hospital materials if the therapeutically more difficult cases of gastric

stump carcinoma (*Stalsberg* & *Taksdal* 1971) and other high stomach cancers are selected for treatment at special hospitals

In case control studies, detailed information on possible risk factors is recorded for each individual case and matched controls On the hypothesis that intestinal and diffuse carcinomas are aetiologically different, it would be logical to compare cases and controls separately in cases of intestinal, diffuse, and other types of carcinoma For this purpose, there should be no objection to the use of autopsy material or metastatic tumour tissue when a surgical specimen from the primary tumour is not available for study

The age-specific I/D ratios of the surgical material of the present study are somewhat lower than those of *Laurén*'s (1965) material from Finland, which was to be expected considering the lower incidence of stomach cancer in Norway (*Doll et al* 1970) The present ratios are also lower than those in the high risk groups in Colombia (*Muñoz et al* 1968) and Connecticut (*Muñoz* & *Connelly* 1971), and higher than the low risk groups in the same areas More surprising and difficult to explain is the finding that the ratios in the present surgical material are substantially higher than those reported by *Muñoz* & *Asvall* (1971) from the same time period in another Norwegian hospital

The distinction between intestinal and diffuse types is not always clear-cut The occurrence of several combined and intermediate tumours, and the change from a combined type in surgical material to either of the main types in autopsy material and *vice versa*, probably indicate that there is a continuous spectrum of morphological types ranging from purely intestinal to purely diffuse Growing in the liver or lymph node sinuses, metastases from diffuse primary tumours frequently showed well formed glandular structures of intestinal type and were classified as intestinal On the other hand, metastases to fibrous and adipose tissues were more frequently of diffuse type than were the primary tumours from which the metastases were derived Data showing that time trends and geographical differences in the incidence of diffuse carcinomas have the same tendencies as those of intestinal type carcinomas, although the changes of diffuse tumours are smaller (*Correa et al* 1970, *Muñoz* & *Asvall* 1971), support the impression that the two types are not entirely separable diseases

Drs *Nubia Muñoz* of the International Agency for Research on Cancer, Lyon, and *John W Berg* of the National Cancer Institute, Bethesda, Md, are gratefully acknowledged for valuable discussions on borderline cases of histological types in the present material Mrs *Bodil Tjade* gave skilful secretarial assistance

This study was supported by U S Public Health Service contract No PH 43-64-499

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Acta path microbiol scand Section A 80, 515 518, 1972

EFFECT OF FRACTURE ON RAT PLATELETS

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Experimental fracture in rats was followed by simultaneous increase in platelet count, platelet stickiness, and occurrence of intravascular platelet aggregates in sections of lungs and kidneys The method used for estimation of platelet stickiness thus seemed to reflect conditions *in vivo*

Trauma is followed by increase in platelet count and by tendency to platelet aggregation (*Hellem* 1968) These changes have been related to thrombosis tendency Platelet aggregation may possibly interfere with the microcirculation and cause irreversible damage in vital organs (*Jörgensen* 1964)

Fracture to some extent may be standardized and has been used as a model of trauma In rabbits an association between increased platelet stickiness and platelet aggregation as seen in histological sections, has been found after fracture (*Gruner* 1971) The aim of the present study was to see if this association was present in another species

MATERIAL AND METHODS

Animals Twenty three white female rats were used Mean weight was 249 g, range 213–294 g They were allowed tap water and pellets (Bjolsen Valsemolle Oslo no 152) Three additional animals died during initial anaesthesia before samples had been taken

Anaesthesia by ether inhalation was used both during blood sampling and production of fracture The anaesthesia lasted for about 5 minutes during which both procedures could be carried out

Fracture of both hindlegs was inflicted in 11 animals using an artery forceps The control group with 12 animals was subjected to sampling only Blood samples were taken immediately before the infliction of fracture and after six days when the experiments were terminated

Platelet enumeration was performed by a slight modification of *Brecher* & *Cronkite's* method (1950) in blood from a transected tail vessel, as previously described (*Gruner* & *Endresen* 1971) The counts reported are thus the mean count as obtained from the first four drops

Platelet stickiness was measured by the platelet reduction rate (PRR) this is the decrease in platelet number, in per cent of initial count, which takes place when the suspensions of blood in ammonium oxalate have been rotated for 1 hour Siliconized tubes were used (*Grüner* & *Endresen* 1971)

Autopsy At termination of the experiments the animals were killed by prolonged ether inhalation Sections of lungs liver, and kidneys were taken for histological examination and fixed and stained according to the MSB method of *Lendrum et al* (1962) The histological examinations were performed 'blindly'

Statistical methods Comparisons Student's t test (P_t) and *Wilcoxon's* two-sample test for paired comparisons (P_W) *Dispersion* The standard error of the mean (SEM) *Two by two tables Fisher Irwin* test (P_{FI}) *Gross error Dixon's* ratio (*Crow et al* 1960) All tests were used two-tailed

RESULTS

With the exceptions mentioned in "methods", all animals survived until termination of the

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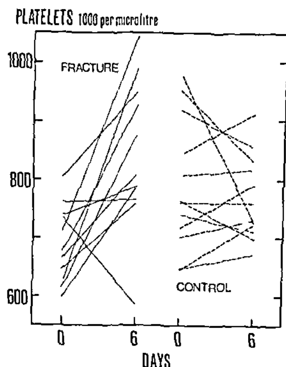


Fig 1 Platelet count before and 6 days after fracture of both hindlegs in rats

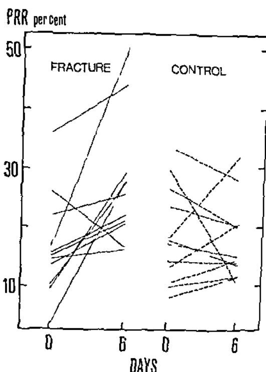


Fig 2 Platelet stickiness, as measured by PRR, before and 6 days after fracture of both hindlegs in rats

TABLE 1 *Platelet Count 6 Days after Fracture of both Hindlegs in Rats, Expressed as per Cent of the Initial Values*

	Fracture	Control	Difference
Number of animals	11	12	
Mean platelet count (per cent)	123 50	98 63	24 87
Standard error of the mean	± 6 89	± 3 26	
P_t	< 0 01 a)		< 0 005 b)
P_W	= 0 01 a)		= 0 01 b)

a) Significance of deviation from 100 per cent   b) Significance of intergroup difference

TABLE 2 *Platelet Stickiness 6 Days after Fracture of both Hindlegs in Rats, Expressed as per Cent of the Initial Values*

	Fracture	Control	Difference
Number of animals	10	12	
Mean platelet stickiness (per cent)	171 89	111 03	60 86
Standard error of the mean	± 27 13	± 12 81	
P_t	< 0 05 a)		< 0 001 b)
P_W	= 0 02 a)		= 0 02 b)

a) Significance of deviation from 100 per cent.   b) Significance of intergroup difference

TABLE 3 *Animals with Platelet Aggregates at 6 Days after Fracture of both Hindlegs*

Injury	No of animals	Lungs	Liver	Kidneys	Lungs, liver, or kidneys
Fracture	11	6	0	5	7
Control	12	0	0	1	1
P_{FI}		= 0 01		= 0 072	= 0 016



Fig 3 Intravascular platelet aggregate in rat lung at 6 days after fracture MSB × 380



Fig 4 An occasional platelet aggregate contained traces of fibrin MSB × 420

experiments Those with fracture seemed enfeebled for the first couple of days

Platelet count was significantly increased six days after fracture (Fig 1, Table 1) The intergroup difference was also significant

Platelet stickiness measured by PRR increased following fracture (Fig 2) This increase and the intergroup difference were significant (Table 2) In one animal PRR increased to an extreme level (about 900 per cent of the initial value) This value was considered an outlier (gross error, $P<0.02$) and was excluded

Platelet aggregates as seen in histological sections (Table 3, Fig 3 and 4) were found significantly more often in the fracture group than in the control group The aggregates were found in the lungs and kidneys but not in the liver Very few of the aggregates contained fibrin, and then only in trace amounts (Fig 4)

DISCUSSION

The increase in platelet count at 6 days after fracture in rat is similar to the increase found by *Latour* & *Renaud* (1966) A period of thrombocytosis is a common post-traumatic event, although its time of appearance seems species dependent In rabbit (*Gruner* 1971) it takes place after 3 days, i e sooner than both in man (*Innes* & *Sevitt* 1964) and rat (*Latour* & *Renaud* 1966)

Platelet stickiness increased following trauma This is a common event in man (*Ham* & *Slack* 1967) and has also been observed in rabbit (*Gruner* 1971) Reports on its time of appearance, however, have been inconsistent (*Hellem* 1968)

Intravascular platelet aggregates are a frequent post traumatic finding (*Eeles* & *Sevitt* 1967 *McKay* 1969, *Gruner* 1972) Their distribution however, shows species variations In rabbits platelet aggregates were

found in lungs, liver, and kidneys (*Gruner* 1971) and in dogs trauma causes platelet trapping mainly in the lungs (*Ljungquist et al* 1971) The present report shows that in rat the aggregates were found in the lungs and kidneys

The scarcity of fibrin within the platelet aggregates may indicate recent and unstable aggregation (*Jörgensen* 1964 *Mustard* 1969) The phenomenon may admittedly be agonal but at least the tendency to platelet aggregation was specific for the traumatized rats Post traumatic transient platelet aggregation however has been observed both by vital microscopy (*Bergent*. 1961) and with ^{51}Cr labelled platelets (*Ljungquist et al* 1971) The effect on the pulmonary microcirculation may be prolonged possibly due to the release of vasoactive substances (*Olsson et al* 1971) Tendency to platelet aggregation has been ascribed to the greater stickiness of newly released platelets (*Ginsburg* & *Aster* 1969) and may be of importance for post traumatic thrombosis and micro embolism (*Mustard* 1969) The importance of increased platelet stickiness as measured *in vitro* is not clear (*Stormorken* 1970 *Ljungquist* & *Bergent* 1971) Increased stickiness measured in rat with the present method seemed to reflect a tendency to platelet aggregation i e *in vivo* conditions thereby confirming previous experiments in rabbits (*Gruner* 1971)

This work was supported by a grant from *Aall's legat*

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Acta path microbiol scand Section A, 80, 519-538 1972

MALIGNANT MELANOMAS OF THE HUMAN CHOROID AND CILIARY BODY

A Comparison of Light Microscopical and Ultrastructural Morphology

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The ultrastructure of fifty malignant melanomas of the choroid and ciliary body has been studied over a period of ten years Based upon twenty tumours embedded in epoxy resins, a comparison between their light microscopical morphology and ultrastructure revealed that the types established by *Callender* have characteristic ultrastructural features The material comprised two spindle A, four spindle B, twelve mixed, one epithelioid and one fascicular tumour The fascicular tumour is the first tumour ultrastructurally studied belonging to this cell type Its ultrastructure may support previous suggestions that the cells have their origin in special parts of the neural crest In view of the prognostic practice of grouping together the fascicular and spindle B tumours, it is remarkable that cells from both these tumours contained cytoplasmic filaments not observed in spindle A or in the epithelioid tumours, and not previously found in choroidal malignant melanomas. The epithelioid tumour was found to be composed of only one type of cell, whereas the tumours of the mixed type appeared to be linked together as a group only by their differing from other types, although features of either spindle or epithelioid cells could be seen to a certain extent in some cells This type probably expresses a certain degree of anaplasia and is not a *mixture* of spindle and epithelioid cells The authors finally sum up the ultrastructural features which they consider characteristic of highly differentiated and of anaplastic malignant choroidal melanomas

Morphological classification based on light microscopical examination of malignant melanomas of the choroid and ciliary body has been attempted a number of times over the last hundred years

For a comparative survey of these classifications reference may be made to (12)

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The classification introduced by *Callender* (4) is still in use by most ophthalmic pathologists, as this classification has been found to be of great prognostic importance The six Callender types are, briefly spindle A, tumours consisting of cells with rather small slender nuclei with ill defined nucleoli, spindle B cells with larger oval nuclei with a prominent nucleolus, epithelioid, characterized by large round or polygonal cells with vesicular nuclei and large nucleoli, *mixed*

tumours consisting mainly of a combination of spindle cells and epithelioid cells, and fascicular, where spindle B cells are arranged in ribbons or in palisades often around blood vessels In addition Callender classified tumours too necrotic to be classified precisely as belonging to the necrotic type

The light microscopical appearance of the types is illustrated in Figs 1–5

Ultrastructural study of malignant choroidal melanomas has been carried out in the last fifteen years in not very many cases (1, 2, 3, 7, 8, 9, 10, 11, 14, 15, 18, 19, 20) No greater success has resulted from this in correlating the light microscopical types with the ultrastructural findings The aim of the present study was to compare the light microscopical and the ultrastructural morphology in the light of the Callender classification and to discuss the possibility of a classification based on ultrastructural features of the tumours

TABLE 1 *Survey of the Material Light Microscopical Classification and Melanin Content*

Type	Melanin content				Number
	Heavy	Medium	Light	Absent	
Spindle A	–	1	1	–	2
Spindle B	–	1	3	–	4
Epithelioid	1	–	–	–	1
Mixed	5	6	1	–	12
Fascicular	–	–	–	1	1
Number	6	8	5	1	20

MATERIAL AND METHODS

The material was collected from 1966 to 1970 and came from patients with choroidal and/or ciliary malignant melanomas Immediately after the enucleation the eyeball was bisected and small pieces of tissue from the central and peripheral parts of the tumour were placed in a drop of ice-cold 5 per cent glutaraldehyde buffered by sodium cacodylate They were sliced in the fixative into pieces of less than 1 mm^3 After fixation in glutaraldehyde for one hour, post fixation was carried out in phosphate buffered osmium tetroxide for half an hour The tissue was dehydrated by ethanol and embedded in Epon 812 (16) Ultrathin sections were cut on an LKB Ultratome III stained with lead citrate (21) and examined in a Philips EM 300 electron microscope with an accelerating voltage of 60 KV

The remaining parts of the tumour were fixed in 4 per cent buffered neutral formaldehyde for 24 hours and embedded in paraffin Several sections from each tumour were used for the light microscopical evaluation In addition to the classification according to Callender the degree of pigmentation was noted Heavy medium light and absent were used as groupings

Ultrastructurally the shape of the cell, the nucleus the nucleolus the mitochondria the ribosomes and endoplasmic reticulum the Golgi apparatus the melanosomes the cytoplasmic matrix any other cytoplasmic structures and the intercellular space were estimated

Although up to now we have collected about fifty malignant choroidal melanomas studied ultrastructurally only material embedded in epoxy resins was used in the present study i e 20 tumours However, when appropriate a comparison was made with the old material and information from this was used in the overall estimation of the types

RESULTS

Light Microscopy

In five cases the ciliary body was involved and in one of these the tumour probably had its origin in the ciliary body

Two tumours were found to belong to the *spindle A type four to the spindle B type* one was epithelioid twelve mixed and one fascicular The melanin content of the tumours is shown in Table 1 The spindle cell tumours contained in general less melanin

Figs 1–5 The different types of malignant melanomas of the choroid and ciliary body classified according to *Callender* (Description see text)

Fig 1 Spindle A EM 23 Lab No 541/66 b Haematoxylin eosin (× 275)

Fig 2 Spindle B EM 22 Lab No 273/66 Haematoxylin eosin (× 275)

Fig 3 Epithelioid EM 33 Lab No 448/69 d Depigmented Haematoxylin eosin (× 275)

Fig 4 Mixed EM 46 Lab No 56/70 Haematoxylin eosin (× 275)

Fig 5 Fascicular EM 25 Lab No 262/67 Haematoxylin eosin (× 275)

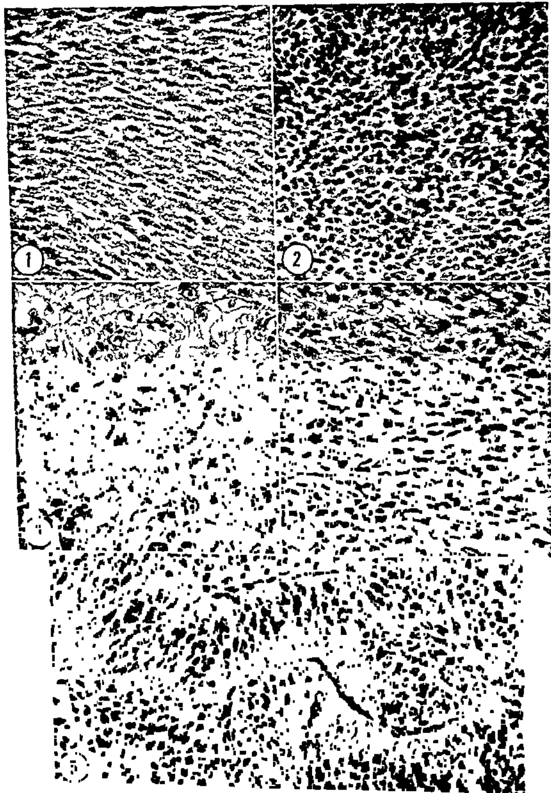

1
2



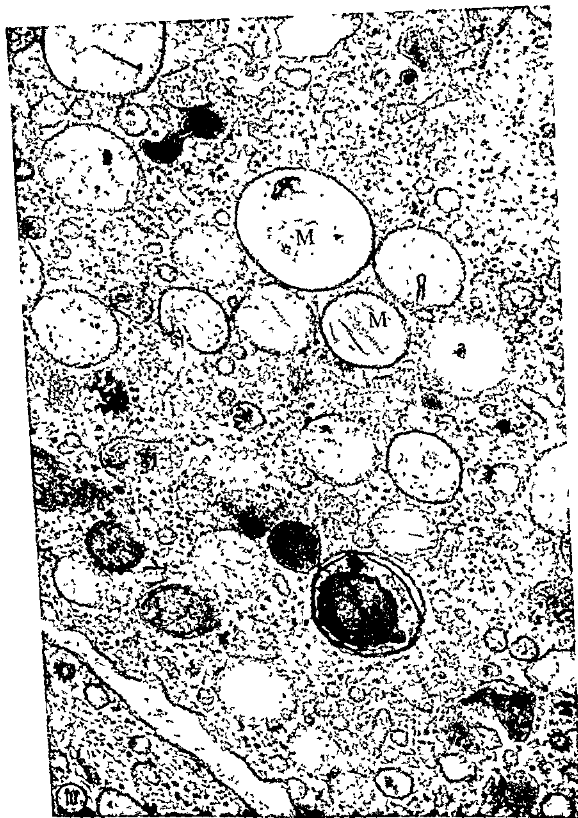
Fig 6 Spindle A tumour Between the closely-packed cells a narrow band of intercellular substance is seen The nuclei (N) are light, and in some cases they contain a single nucleolus (NU) In the cytoplasm the mitochondria (MI) appear swollen with loss of internal structure (× 9,300)

Fig 7 The nucleoli of the spindle A nuclei were in many cases placed peripherally opposite a large pore in the nuclear membrane (PO) Note the intercellular substance (arrow) (× 16,500)

Fig 8 Spindle A tumour Two cells with nuclei (N) and separated by a narrow intercellular space are seen One cell contains a well developed Golgi complex consisting of 4 5 flattened cisterns and a number of light vesicles (GC) (× 28,500)






M
M

than the mixed and the epithelioid tumours

Sections of each type are illustrated in Figs 1–5

Electron Microscopy

The two *spindle A* tumours were ultrastructurally identical They were composed of closely placed uniform cells The shape was difficult to evaluate because of the thin sections but undoubtedly the cells had one or more cytoplasmic processes (Fig 6)

The nuclei were large compared with the cytoplasmic volume and their outline rather regular, with one or two indentations They were poor in chromatin which was diffusely scattered apart from a narrow (200–300 Å) zone against the inner nuclear membrane Some nuclei had a nucleolus seen distinctly because of the very light nucleus In several cases the nucleolus was lying peripherally in continuity with the above mentioned narrow border of chromatin and it was striking that precisely in this place a large pore in the nuclear membrane was seen (Fig 7)

The matrix of the cytoplasm was moderately electron dense and a large number of free ribosomes were seen The mitochondria were large almost vesicular with indistinct crests and a very light matrix (Figs 6 7)

The Golgi apparatus was well developed in many cells (Fig 8) Melanosomes were scarce and were seen as small vesicles round or oval with a diameter varying from 0 06 μ to 0 14 μ

The narrow intercellular spaces were filled by a homogeneous electron dense material (Figs 6 7) which was not observed in any of the other types examined

The shape of the cells in the four *spindle B* tumours was similar to that of the spindle A tumours

The nuclei were smaller than the spindle A nuclei the indentations were more numerous and deeper the chromatin content larger

The chromatin was condensed into irregular shaped lumps of varying size lying diffusely in the nucleus and along its inner membrane The nucleoli were large and were present in nearly all cells

The cytoplasm was in general rich in free ribosomes while only a few elements of rough surfaced endoplasmic reticulum (RER) could be detected The Golgi apparatus was very well developed (Fig 9), composed of four to six flat cisterns surrounded by numerous vesicles with a light content

The mitochondria were rod shaped with regular crests and a homogeneous matrix A number of the cells contained mitochondria with dense granules about 100 Å in size At higher magnification these granules were found to be composed of smaller particles 50–70 Å in diameter (5) Cells with these mitochondrial granules did not significantly differ from the other tumour cells Melanosomes were more frequent than in the spindle A tumours They were more polymorphous with a diameter from 0 05 μ–1 μ and the degree of melanization varied greatly (Fig 10) No correlation between the size of the melanosomes and their content of melanin was found Some of the largest melanosomes had no melanin at all but remnants of the protein matrix on which the tyrosinase molecules are assumed to be localized (22) could be seen interiorly (Fig 10) In three tumours the cytoplasm contained long thin filaments 30–60 Å in thickness, of indeterminable length and without periodicity They ran mainly in bundles of varying thickness in and out between the various cytoplasmic elements (Fig 11) The intercellular spaces were broader than those of the spindle A tumours and contained few collagen fibrils

The cells of the *epithelioid* tumour were all large with a high nuclear cytoplasmic ratio (Fig 12) Only one type of cell was found The shape was remarkable the plasma

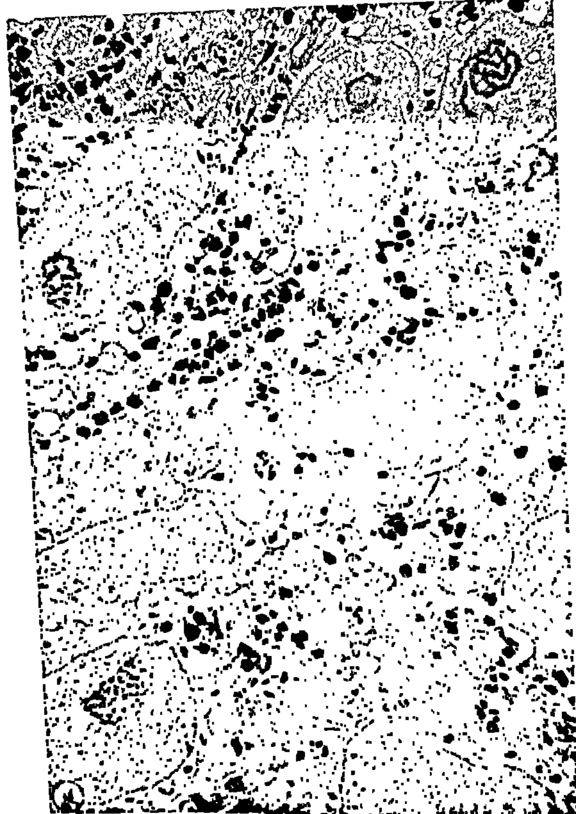
Fig 9 Spindle B tumour Several fragments of cells are seen indicating an elongated cell shape GC Golgi complex M melanosomes (× 36 000)

Fig 10 Spindle B cell showing the polymorphism of the melanosomes (M frequently found in this type of tumour (× 71 000)

Fig 11 In three of the spindle B tumours examined thin filaments (arrow) were present in the cytoplasm (× 71 000




M
M
12
14



Current No	29	30	32	35	37
Pigment	heavy	medium	light	heavy	heavy
Shape of cells	spindle	spindle	spindle	spindle	spindle
Nuclei	as spindle B	as spindle A	as spindle B	as spindle B	as epithel oid
Free ribosomes	moderate	few	moderate	moderate	moderate
Mitochondrial shape	rod	sphere	rod	rod	rod
Ergastoplasma (RER)	moderate amounts	moderate amounts	moderate amounts	small amounts	moderate amounts
Golgi complex	moderate	moderate	moderate	moderate	small
Glycogen	absent	absent	absent	absent	absent
Filaments	absent	absent	absent	absent	absent
Intercell spaces	small	large	small	small	large
Collagenous fibres	absent	moderate amounts	small amounts	absent	small amounts

membrane being drawn out into numerous long processes, interdigitating with corresponding processes from the neighbouring cells In this way the intercellular spaces were reduced to narrow clefts (Fig 13)

The nuclei were unusually irregular with long processes and deep indentations (Fig 12), a morphology which on light microscopy made the nuclei appear to contain lumps of eosinophilic material The nuclei were poor in chromatin but had large nucleoli

Small elements of RER were found, and the mitochondria were numerous and polymorphous The cytoplasm was rich in melanosomes and the content of each melanosome was larger than in the above mentioned tumours Most cells had glycogen particles in their cytoplasm (Fig 12)

Each of the twelve tumours classified as *mixed* cell type appeared to be composed of its own type of cell with an ultrastructure different from spindle cells and epithelioid cells, although features from either type could be more or less dominant

The cell type, however varied somewhat from tumour to tumour so that no tumours appeared to be completely identical in their ultrastructure

The shape of the cells was either oval or ellipsoid (Fig 14) In one tumour (No 40) however, the cells were nearly spherical as in the epithelioid tumour (Fig 15), but the interdigitating cytoplasmic processes were absent The nuclei resembled in most cases those of the spindle B tumours chromatin rich and with large nucleoli and further with lumps of chromatin in the nucleus and along the inner nuclear membrane Nuclei resembling those of the epithelioid tumour *i e* large light heavily indented and with a

Fig 12 Epithelioid tumour cell containing melanosomes (M) and glycogen particles (G) The nuclear membrane (NM) is extensively folded (× 32 000)

Fig 13 Cytoplasmic interdigitation between two adjoining tumour cells of the epithelioid type (× 35 000)

Fig 14 Survey picture of a tumour of the mixed cell type In this case the nuclei highly resemble those of spindle A (× 9 000)

Tumours of the Mixed Cell Type
Features

38	39	40	41	42	45	46
heavy	heavy	medium	medium	medium	medium	medium
spindle	spindle	epithelioid	spindle	spindle	spindle	spindle
as epithelioid	as spindle B	as spindle A	as spindle A	as spindle B	as spindle B	as spindle B
moderate	few	moderate	few	few	few	few
sphere	sphere	rod	rod	sphere	sphere	sphere
small amounts	small amounts	small amounts	moderate amounts	moderate amounts	moderate amounts	small amounts
small	small	moderate	extensive	extensive	small	moderate
absent	absent	absent	absent	present	absent	absent
present	*absent*	*absent*	*absent*	*absent*	*absent*	*present*
small	large	large	small	large	large	small
absent	moderate amounts	large amounts	absent	absent	absent	large amounts

prominent nucleolus, were found only in one case (No 37) Nuclei resembling spindle A nuclei were found in three tumours (Nos 30, 40, 41)

In about half the tumours the mitochondria were well proportioned with a moderately electron dense matrix, while the rest showed various peculiar characteristics bizarre shapes swelling irregular crests, sometimes defective in their central parts and light in some cases absent, matrix

The melanosomes were also very varying in size shape and melanin content Instead of being a mixture of spindle cells and epithelioid cells this type is therefore composed of various cells expressing a certain degree of anaplasia For comparison of the various features see Table 2

The cells of the *fascicular* tumour were uniform and closely placed The nucleus was vesicular and centrally placed The chromatin content was sparse and evenly distributed apart from a narrow zone along the inner nuclear membrane No nucleoli were observed The nuclei resembled those of the spindle A tumours, but were even more regular

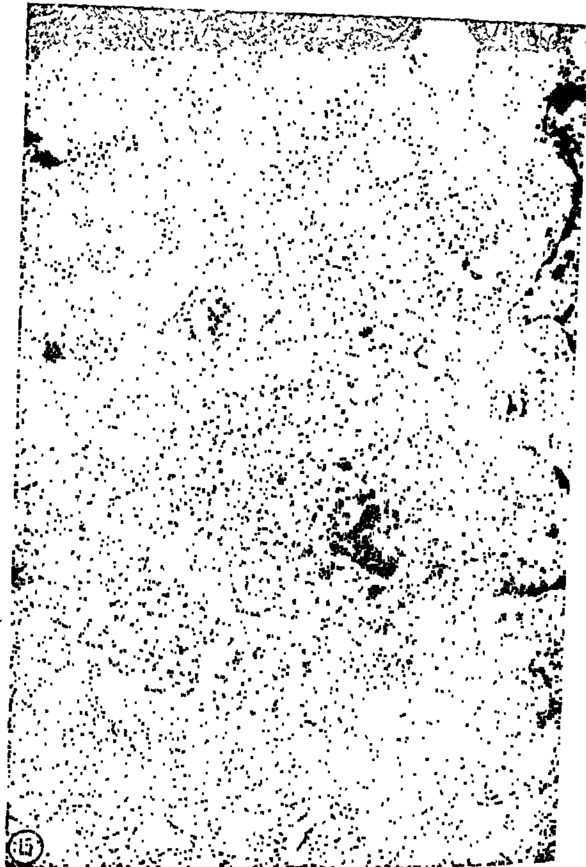
The cytoplasm was remarkably light and had numerous free ribosomes and a small Golgi apparatus as well as rod shaped mitochondria with distinct substructure (Fig 16)

Although no melanin granules were seen on light microscopy, small melanosomes of up to 0 1 μ were observed Each contained numerous very electron dense particles about 50 Å in size (Fig 17) A few filaments indistinguishable from those of the spindle B tumours were found in the cytoplasm (Fig 17)

Fig 15 Tumour of the mixed cell type with nucle resembling those of spindle B Note the large ac cumulations of glycogen particles (G) in the cyto plasm (× 15 500)

Fig 16 Part of a cell from the fascicular tumour Unlike the spindle tumours, the nucleus has n indentations and the distribution of chromatin i more uniform (× 32,500)

Fig 17 Cell from the fascicular tumour In th cytoplasmic matrix, thin filaments (arrow) of th same size and distribution as in some of the spind B tumours are seen M melanosomes (×66 500)




17

DISCUSSION

François et al (7 8 9 10 19) described the ultrastructure of a pigmented and an unpigmented spindle B tumour and found for the most part the same ultrastructure in both They contained together with features also found in our spindle B series but in contrast to our findings a well developed smooth surfaced endoplasmic reticulum (ER) and larger formations of rough surfaced endoplasmic reticulum (RER) consisting of concentric lamellae In this respect it may be of interest that similar annulate lamellae have been found in human melanoma cells in cell culture (probably skin melanoma but not stated) (17) In their later study of twenty malignant melanomas of the choroid (10) the above mentioned writers did not specify the cell type but they stated that no pure epithelioid type was studied

Hogan & *Feeney* (11) studied ten malignant melanomas of the choroid and ciliary body two spindle B seven mixed and one epithelioid The most striking difference from our findings was the observation of two types of cell in the epithelioid tumour—a dark and a light type but these writers stated that the cytoplasmic components were similar in the two types only the quantity of cytoplasmic organelles especially the number of free ribosomes accounting for the difference Further, they found smooth and ... al

plasma membrane

In their spindle B cells they found no chromatin layer at the inner nuclear membrane and no free ribosomes both findings in contrast to ours On the other hand their spindle B tumours exhibited the same spiral shaped RER as seen by *François et al*

In no previously examined spindle B cells were fine filaments as seen in our spindle B cells described *Hogan* & *Feeney* found it more difficult to distinguish spindle like and epithelioid like cells in the mixed tumours because of their more cytoplasmic variation which was also our experience However they tried to distinguish between three classes of spindle cell and epithelioid cell in the mixed tumour We found it very difficult to make this distinction and found no cells in our twelve mixed tumours which fitted into the pattern of either spindle cells or epithelioid cells as seen in our spindle cell tumours or the epithelioid although some cells had features suggesting spindle cells and others features suggesting epithelioid cells

Kroll & *Kuwabara* (14) published the study of representative non pigmented regions of four spindle B and two epithelioid melanomas aiming especially at a comparison of spindle and epithelioid cells

As in our spindle B cells the cytoplasm was rich in free ribosomes and only fragments of RER were seen In contrast to us they occasionally observed desmosomes They confirmed the findings of *Hogan* & *Feeney* that the epithelioid tumours have light (lucent) and dark (dense) cells and that the difference was only caused by the varying quantity of identical organelles In our opinion this distinction appears to be artificial

In the dense cells, but not in the lucent ones they found mitochondria with osmiophilic granules within their matrix similar to our findings in mitochondria of spindle B cells to judge from the illustrations A difference between the two cell types of the epithelioid tumour on this basis is therefore not justified Regarding the nature of such granules reference may be made to (5)

The publication of *Okuda et al* (18) of the ultrastructure of two spindle B tumours confirmed the previous findings mentioned above that spindle B cells may contain free ribosomes predominantly or RER predominantly

In a recent study of surface properties of human melanoma cells by *Lapis* & *Radnot* (15) these writers in their study used two choroidal malignant melanomas composed of spindle A cells The cells had fairly regular nuclei and the cytoplasm was rich in free ribosomes Of special interest in relation to our findings of a homogeneous electron dense intercellular material (Figs 6 7) is their demonstration by ruthenium red of a fairly even

layer of electron opaque deposits on the surface of the cells, in some areas giving the intercellular spaces the appearance of being *completely filled* This ruthenium red positive substance is considered to be a polysaccharide It is difficult to say if the intercellular material in our spindle A tumours is of this nature This is probably not the case, because several writers (15) have found that malignant cells display a thicker polysaccharide coat than benign cells, and an intercellular substance similar to that of our spindle A cells was not observed by us in any of the more anaplastic types A ruthenium red study of the surface of these types would be of great interest

Based on our present experience, the different types may be briefly characterized as follows

Spindle A Narrow intercellular spaces filled by electron dense material Rather large nuclear cytoplasmic ratio Regular nuclei poor in chromatin and sometimes without observable nucleolus Numerous free ribosomes Large, irregular mitochondria and few melanosomes

Spindle B Broader intercellular spaces with collagen fibrils Smaller, more irregular nuclei than spindle A, chromatin in lumps, nucleolus rather large Numerous free ribosomes and small elements of RER Rod-shaped mitochondria, moderate number of melanosomes, and cytoplasmic filaments

Epithelioid Interdigitating processes of the plasma membrane narrowing the intercellular space Large irregular nucleus poor in chromatin with a large nucleolus few free ribosomes and small amounts of RER, large melanin-rich melanosomes and cytoplasmic glycogen

Mixed Mainly broad intercellular spaces Nuclei rather irregular with lumps of chromatin Large nucleoli Nuclei predominantly similar to the spindle B nuclei The mitochondria, ribosomes, RER and melanosomes very varying in number and morphology from tumour to tumour (Table 2) No correlation between the single combinations of organelles could be found Although forms transitional to spindle cells and epithelioid cells may be established, it is our experience that the cells of the mixed tumours have their own characteristics, and that they cannot be described as a mixture of spindle and epithelioid cells

Fascicular Narrow intercellular spaces Large, regular, vesicular nuclei without observable nucleolus and very sparse chromatin Moderate number of free ribosomes, rod shaped mitochondria, few small, lightly melanized melanosomes, and cytoplasmic filaments

By way of comment on the above, the criticism may be made that the mitochondria in the spindle A tumours and some mixed tumours were almost vesicular or polymorphous with indistinct crests, and that this may be a sign of bad fixation But we do not believe this to be a fixation artifact, as the perinuclear cisterns in these cases were well preserved, and these are usually the first structures affected by insufficient fixation The fascicular tumour is as far as we can see the only one of this type ultrastructurally examined It is very interesting that filaments similar to the filaments in the spindle B tumours were found in the fascicular tumour as this may support the assumption that tumours of the fascicular cell type are composed of spindle B cells but in a special pattern, and that the two types can be grouped together prognostically

The very regular shape of the nucleus and the cell as a whole, with its light cytoplasm and few small melanosomes, reminds the writers of the small oval cell with little branching that *Feeney & Hogan* (6) found in the normal human choroid together with a large branched melanin-rich melanocyte The tendency of the fascicular tumour to form neural plate- and neural tube like formations may suggest an origin of its cells (and of the small cell in the normal choroid) close to the neural tube (12)

Although in a preliminary study of our material we had the impression that only three types could be ultrastructurally differentiated (13), the complete study of our

present material has shown that the types established by Callender appear to have their specific ultrastructural characteristics A study of more material may, however, change our views

Although the different types have the above-mentioned characteristics, we would, according to our present knowledge, stress the following features as the more important in evaluating the degree of differentiation on an ultrastructural basis The shape of the cell, although this may be difficult to estimate because of section thickness, the size and shape of the nuclei and nucleoli, the number of mitochondria, not their size or shape, the presence or absence of cytoplasmic filaments, glycogen granules in the cytoplasm as an expression of immaturity We do not consider the amount of ribosomes—free or bound— of any value, although the presence of numerous free ribosomes may indicate a rapid cell division and abundant RER a controlled synthesis of protein Both spindle and epithelioid cells may contain each of these states In the same way, melanosomes may be large or small and more or less melanized, but all in all the more anaplastic cells appear to be the more pigmented It must be pointed out that dedifferentiation may express a disturbance which affects only one or only a few of the many cell functions

Based on these observations and assumptions the tumours we consider the more differentiated are those with closely-placed fusiform cells, with homogeneous material or collagen fibrils intercellularly, regular nuclei with small or non-observable nucleoli a moderate number of mitochondria cytoplasmic filaments and perhaps few melanosomes, whereas large cells with broad intercellular spaces or numerous interdigitation processes of the plasma membrane, with large irregular nuclei and prominent nucleoli, with numerous mitochondria cytoplasmic glycogen granules and perhaps numerous large melanosomes are considered as more anaplastic

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This work was supported by a grant from the *Danish National Anti Cancer League*

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Acta path microbiol scand Section A, 80, 539-547, 1972

ENZYME STUDIES IN RATS WITH EXTRA-HEPATIC BILIARY OBSTRUCTION

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Ligation or transsection of the common bile duct in rats was followed by a histochemically demonstrated increase of alkaline phosphatase reaction in the liver from the 1st postoperative day, and centrolobular loss of canalicular adenosine triphosphatase reaction from the 5th day The peripheral canaliculi appeared thickened and irregular The acid phosphatase reaction was increased in hepatocytes and Kupffer cells Gamma glutamyl transpeptidase became positive in an increased number of bile ducts and ductules With Nitro BT as substrate the formazan products of succinic dehydrogenase and monoamine oxidase changed from regular, coarse sinusoidal granules into irregular masses along the sinusoids and the finely granular hepatocytic products became reduced. Serum alkaline phosphatase and gamma glutamyl transpeptidase reached a maximum at 1 day and 2 weeks, respectively, after operation

A number of reports on enzyme histochemical studies in rats with biliary obstruction have been published during the last decade (6, 8 9, 10 13, 14 16, 17, 19, 20, 21) This paper concerns a histochemical investigation of 7 enzymes representing the different cell organelles in the liver of rats with extrahepatic biliary obstruction Alkaline phosphatase and gamma glutamyl transpeptidase in hepatic tissue were compared with corresponding values in the serum, where the levels are known to rise in biliary obstruction

MATERIAL AND METHODS

102 male, albino rats (weight 190-300 g, age 6 12 months) were used In 29 rats the common bile duct was ligated and in 42 it was divided between double ligatures 28 rats served as controls, half of which were subjected to sham operations In addition, 3 rats were fasted 24 hours before the experiment, 2 of which were operated upon with transsection of the bile duct, while the 3rd was subjected to sham laparotomy These rats were allowed to survive for only 1 day Blood samples for enzyme analyses were obtained by heart puncture from all the animals at the beginning of the experiment and again in association with the excision of biopsy specimens about 1×0 5 cm in size from the ventral, right parts of the liver The operations and the heart punctures were performed under ether anaesthesia in the morning with the animals fasting Biopsy specimens were excised from groups of 2-3 experimental animals and 1 control 4-6 hours, 1, 2, 3 4 days and 1, 2 3, 4, 5, 6, 7 weeks, respectively after the bile duct operation

Laboratory studies of the blood samples

1 Serum alkaline phosphatase was determined by a modification of the method of *Bessey et al* (4)

2 Serum gamma glutamyl transpeptidase was determined by a modification of the method of *Szewczuk & Orlowski* (18) using N-(gamma-L-glutamyl)-alpha-naphthylamide as substrate

Received 5 1 72

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serum activity rose after 1 day, and values 10 to 20 times normal were noted in rats killed 2 weeks after transsection of the common bile ducts while most rats subjected to ligation of the duct had normal values for at least 4 weeks after the operation (Fig 1)

The control rat deprived of food for 24 hours before the 1st heart puncture and then sham operated upon and killed the next day showed no difference in serum activity of alkaline phosphatase or gamma glutamyl transpeptidase between the two examinations, and the values were within the same range as those of the other controls The two fasted and operated rats showed an increase of the two enzymes of roughly the same size as the other rats killed 1 day after the operation

Histology

The early histological changes were similar in all the 73 experimental animals Thus after 4–6 hours no microscopical changes could be seen in the liver Among 21 rats killed 1–5 days after the operation 11 showed small hepatocellular necroses with a leucocytic reaction Such necroses were rarely seen in animals killed later Centrolobular fatty changes were common From the 3rd day onwards the portal zones appeared more prominent with some inflammatory cells and high epithelium in the bile ducts From the 4th day ductules had begun to proliferate and after 2 weeks they completely surrounded the hepatic lobules After 3 to 5 weeks the hepatic parenchyma consisted of an irregular mixture of hepatic cells and ductules The hepatic cells varied in size and their nuclei were prominent and hyperchromatic These advanced changes were found in rats operated upon with transsection of the common bile duct but only in a few of those in which the duct had been ligated Among those belonging to the latter group and killed 2 7 weeks after the operation some showed a slight increase of the periportal connective tissue and proliferating ductules while others showed no pathological changes Only few rats showed histological changes in the kidneys These changes consisted of eosinophilic cylinders in the tubules and small infarcts after long standing biliary obstruction The enlarged spleens were of normal microscopical appearance

Histochemistry

Succinic dehydrogenase In control rats finely granular formazan was deposited in all the hepatic cells often more densely in the periphery of the lobule, while coarsely granular formazan was localized along the sinusoids From about the 2nd day of biliary obstruction the coarse granules were distributed



Fig 2 Monoamine oxidase
a Control × 300
b Biliary obstruction for 2 days × 300



more irregularly along the sinusoids In animals killed later, hepatic cells filled with finely granular formazan were seen near the growing portal zones

Monoamine oxidase The control rats invariably showed an even distribution of finely granular formazan within hepatic cells and coarsely granular pigment along the sinusoids (Fig 2a) The enzyme reaction pattern of experimental rats were about the same as that of the succinic dehydrogenase reaction (Fig 2 b)

Nonspecific esterase In control rats all the hepatic cells stained uniformly In rats killed 3–5 weeks after the operation some hepatic cells stained more intensely Most of these cells were near to growing portal areas

Acid phosphatase In control rats, pericanalicular rows of granular enzyme reaction products were observed throughout the hepatic plates with stronger staining in the periportal third where also most of the enzyme positive Kupffer cells were found (Fig 3a) From about the 4th day the reaction demonstrated more numerous Kupffer cells and more pronounced hepatocytic lysosomes delineating canaliculi as empty spaces (Fig 3b c) This increased staining in hepatocytes persisted also in late stages of biliary obstruction when the normal lobular structure had been destroyed A weakly positive acid phosphatase reaction in inflammatory cells appeared in the growing portal areas

Alkaline phosphatase In the controls the product was localized around bile ducts and arteries and appeared as scattered dots in the portal connective tissue and the sinusoidal walls There was also a weak continuous reaction along the sinusoids The blood vessels occasionally contained enzyme positive leucocytes Only in a few animals did canali

Fig 3 Acid phosphatase
a Control Central vein to the left × 181
b Biliary obstruction for 5 days Central vein to the right × 184
c Biliary obstruction for 2 days × 300

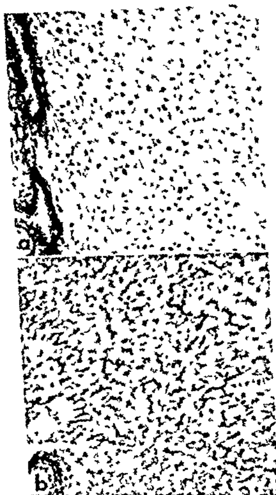


Fig 4 Alkaline phosphatase
a Control Portal zone to the left × 144
b Biliary obstruction for 3 days Portal zone to the left × 144

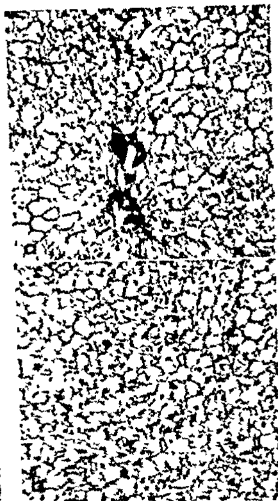


Fig 5 Adenosine triphosphatase
a Control Portal tract in the lower part × 184
b Biliary obstruction for 2 days × 184

culi stain mainly periportally (Fig 4a) Already after 1 day's biliary obstruction the alkaline phosphatase reaction increased in the corresponding areas which were positive in the control rats and sometimes with canalicular staining throughout the lobules The perisinusoidal staining was not conspicuously increased until the common bile duct had been obstructed for some time (Fig 4b)

Adenosine triphosphatase showed a canalicular pattern throughout the hepatic lobules as well as staining of connective tissue around vessels and bile ducts of portal regions in the control rats (Fig 5a) From about the 4th day there was a patchy mainly centrolobular loss of positive canalicular structures (Fig 5b) A distinct sinusoidal reaction occurred in the same regions and sometimes the reaction products marked the border between hepatic cells The remaining stained canaliculi were coarse and irregular After 2–5 weeks biliary obstruction the canalicular pattern was barely discernible while the staining around ductules and vessels in the growing portal areas and in the periportal sinusoids was very prominent

TABLE 1 *Review of Enzyme Histochemical Investigation of Hepatic Tissue from Rats with Biliary Obstruction*

Authors	Operation	Duration of experiment	Enzyme reactions	Results in summary
Cameron & Muzaffar, 1958	bile duct excision	1 hour to 11 weeks	alkaline *phosphatase*	1–2 days necroses with loss of *enzyme* 4 days–11 weeks increased enzym reaction of bile duct epithelium, canaliculi, adventitia of blood ves sinusoids
van Wersch, 1963	bile duct ligation	1–14 days	alkaline phosphatase	increased enzyme reaction localize connective portal tissue, blood ves walls, sinusoids, Kupffer cells, canaliculi, cell membrane of hep cells
Wachstein & Meisel, 1958	bile duct ligation	1 day to 3 weeks	adenosine triphosphatase	first increase in canalicular stain later increasing sinusoidal staining loss of canaliculi in patches
Schatzki, 1963	duct excision	2 hours to 36 days	adenosine triphosphatase	loss of canalicular and increase of sinusoidal staining
Wegman & Charbonnier, 1964	duct ligation	8–10 days	adenosine triphosphatase	decreased canalicular activity
Goldfischer, Arias, Essner & Novikoff, 1962	bile duct ligation	24 hours	alkaline phosphatase adenosine triphosphatase acid phosphatase	increased activity in canaliculi sinusoids and adjoining cell surface decreased activity in periportal canaliculi dilatations, fragmentatio twisted branchings of the canaliculi increased numbers of lysosomes distributed throughout the cell
Birns, Masek & Auerbach, 1962	bile duct ligation (around a rubber *tubing*)	1–3 (–6) days	alkaline phosphatase adenosine triphosphatase acid phosphatase	1–2 days increase localized to canaliculi 3 days *loss of* canalicul staining (At release of the biliary obstruction first increase, within 3 days decrease to the normal picture 1 3 days decrease of canalicular activity (After release, normal reaction within 3 days) 2–3 days increase of positive sinusoidal lining cells
Krstulović van Damme Desmet, 1968	bile duct excision	1–6 days	alkaline phosphatase adenosine triphosphatase acid phosphatase	12 36 hours increase After 36 hours decrease After 1 day decrease of canalicular activity *12–48 hours* displacement in a broader pericanalicular *zone* After days numerous large, positive Kupffer cells

uthors	Operation	Duration of experiment	Enzyme reactions	Results in summary
Iajdu & oraker 1964	ligation of hepatic duct to median or left lobes	4–30 days	adenosine triphosphatase succinic dehydrogenase	first loss of canalicular stainir later increase in sinusoidal star on the 4th day increase peripo and around necroses weak react centrolobularly
ohen, 1965	duct excision	4–19 days	succinic dehydrogenase	increase with the normally stro periportal reaction spreading cen until an even strong reaction appeared throughout the lobule
ietlén 1965	the paper was not available			
hou & Gibson 971	duct excision	2 hours–60 days	alkaline phosphatase	within 24 hours increase localize canaliculi sinusoids connective portal tissue
			adenosine triphosphatase	48 hours increase in canaliculi A 7 days decrease to total loss of canalicular staining
			acid phosphatase	at 24 hours large intensively sta granules in necrotic areas (Charcot Gombault necroses) Fr the 5th day larger clumped and irregularly scattered granules in liver cells
			succinic dehydrogenase	foci of necrosis unreactive otherwise small changes

Gamma glutamyl transpeptidase revealed no activity in control rats except occasionally in the epithelium of the bile ducts In the experimental rats the enzyme showed a weak reaction in the epithelium of proliferating bile ducts and ductules but no activity in canaliculi or sinusoids

All the enzyme reactions were negative in the *necrotic areas* seen in rats killed 1–5 days after the operation In adjacent parenchyma the canalicular alkaline phosphatase reaction was increased Some of the rats with a ligated common bile duct killed at 2–7 weeks and showing a normal histological picture also had a normal enzyme histochemical pattern while some exhibited an increased reaction for alkaline and acid phosphatase and some a patchy loss of canalicular adenosine tri phosphatase

DISCUSSION

The morphological changes observed after ligation or transsection of the common bile duct were in good agreement with those found in earlier investigations (7 11 12) It is thus well known that after ligation the common bile duct becomes recanalized spon taneously with regression of the morphologi cal hepatic changes Increased staining for alkaline and acid phosphatase as well as loss of canalicular adenosine triphosphatase per sisted in some rats with recanalization of the common bile duct and otherwise normal histology Enzymatic normalization thus ap pears to lag behind morphological recovery Experiments on enzymatic histochemical changes in rats during biliary obstruction have been reported (5 6 8 9 10 13 14 16 17 19 20 21) Some of these investigations are summarized in Table 1 Alkaline phos

phatase activity was generally found to increase while adenosine triphosphatase proved to decrease in canaliculi, but to increase in sinusoids The observation by *Birns et al* (6) and *Krstulović et al* (16) of a decrease of hepatic alkaline phosphatase after its initial increase in biliary obstruction could not be confirmed in this experiment

The reports of succinic dehydrogenase activity differ from one another This may be due to differences in the methods used and the difficulties in interpretation of the staining products, i e, formazans The coarsely granular formazan observed in this study might be due to absorption of tetrazolium by fat and reduction by coenzyme Q Using tetra-Nitro Blue as well as Nitro Blue tetrazolium, we have observed fine and coarse formazan granules in hepatic tissue, even though coarse granules appeared less numerous with the former tetrazolium salt For further discussion of this problem the reader is referred to a recent report by *Kakari* (1970)

The changes found in the activity of serum alkaline phosphatase in the rats during extra hepatic biliary obstruction were in agreement with the findings by *Dalgaard* (1947) though the rats in the present experiment were not fasted for a long time before the operation

In investigations on dogs (2 3) it was found that alkaline phosphatase activity of hepatic tissue, but not of serum increased after nembutal anaesthesia and sham laparotomy, but this is obviously not the case in rats after ether anaesthesia and laparotomy

A more extensive discussion will be given in a separate report where cholestatic human dog and rat livers will be compared in respect of the histochemical behaviour of their enzymes

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Acta path microbiol scand Section A, 80, 548-556, 1972

QUANTITATIVE DETERMINATION OF DEHYDROGENASE ACTIVITY IN TISSUE SECTIONS AND TISSUE HOMOGENATES

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The dehydrogenase activity in tissue sections was demonstrated by means of the ditetrazolium salt Nitro Blue Tetrazolium It was found to be reduced to formazan compounds which, when precipitated in the tissues, indicate the enzyme activity The same method can be employed for an estimation of the dehydrogenase activity in tissue homogenates The formazan compounds are dissolved and the amount determined colorimetrically The accuracy of the results depends on a complete dissolution of the produced formazan compounds A method fulfilling these requirements is described It was shown in a series of experiments that the produced amount of formazan indicates a corresponding enzyme activity (dependent on the added specific substrate) The reproducibility of the results is demonstrated and the influence of the pH values on the enzyme activity is discussed The necessity of including control sections and blanks is illustrated Comparative studies of semiquantitative (histochemical) and quantitative (colorimetric) measurements gave concordant results Finally, it was shown that microscopy of tissue sections stained for dehydrogenase activity affords a possibility of estimating the intensities of the enzyme activities

The commenly employed methods for determination of dehydrogenase activity in homogenates and tissue fluids are based on spectrophotometric and fluorimetric principles

The activity of co enzyme-dependent dehydrogenases is most easily demonstrated by measuring the altered extinction to occur in response to reduction of the co-enzyme The co-enzyme-independent dehydrogenase activity is measured by means of a colorimetric determination of the reduction of an added tetrazolium salt to formazan

Using the histochemical procedure the tetrazolium salt method is employed for demonstrating the activities of both co enzyme-dependent and co-enzyme independent dehydrogenases The formazan granules produced at the reduction become deposited *in situ* in the tissues, a process which marks the enzyme activity

The present paper describes a series of experiments showing the usefulness of the ditetrazolium salt Nitro-blue tetrazolium as an indicator in the demonstration of dehydrogenase in both tissue sections and homogenate

The object of this study was to find answers to the following two questions

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1 Can reduction of the tetrazolium salt to formazan deposited *in situ* in the tissue sections be interpreted as a quantitative and specific indication of an existing enzyme activity, or are the dye precipitations in part non enzymatically determined?

2 Will a semiquantitative assessment of dehydrogenase activity in tissue sections give results comparable with values obtained by corresponding analyses of tissue homogenates?

MATERIAL AND METHODS

Tissue blocks of rat liver are frozen down immediately after the excision in isopentane, by immersion in an acetone/CO_2 snow cold mixture (−80°) The dehydrogenase activities are thereafter measured partly in homogenized tissue, and partly in tissue sections

The following enzyme systems were tested NADH tetrazolium reductase (NADH Tr), lactic acid dehydrogenase (LDH), succinic acid dehydrogenase (SDH) and glucose 6 phosphate dehydrogenase (G 6 DH)

Homogenate Experiments

The ditetrazolium salt Nitro-blue tetrazolium (N BT) was used as indicator in the determination of the activities of both co-enzyme dependent and co enzyme independent dehydrogenases A tissue sample was homogenized in a Potter Elvehjem glass homogenizer, after addition of 0 2 M phosphate buffer, pH 7 4 80 microlitres of buffer were added per milligram tissue

The incubation medium was prepared so as to contain the same specific substrates co-enzymes and indicator as described in the following section on histochemical demonstration of dehydrogenase activity To each sample were added 0 4 ml of homogenate and 1 ml of incubation medium Blanks were incubated without adding specific substrate but otherwise under identical conditions The enzyme activity was interrupted by adding 300 microlitres of 1N hydrochloric acid The formazan produced was extracted as follows The reaction mixture was added to 2 ml of distilled water after which followed gentle shaking Then 3 ml of chloroform was added and the mixture was shaken vigorously (mechanically) for 30 minutes The [illegible]

[illegible] chloroform settled basally in the test tube under a layer of distilled water which prevented evaporation The colorimetric measurement was undertaken with a Beckmann's photometer at wavelength about 540 nm

The Histochemical Methods

The method has been developed by *Thomas & Pearse* (17) Fresh, frozen tissue sections, about 8 my thick are cut on cryostat (Slee, Pearse) The sections are thereafter incubated at 38° for 5 10, 20 and 30 minutes in the following media

[illegible]

ml of distilled water To this are added the specific substrates, all calculated per ml of the above solution

NADH, tetrazolium reductase 2 mg NADH (Sigma, D 7380) in 0 1 ml of water

Lactic acid dehydrogenase 0 1 ml of 1N sodium DL lactate (Sigma, L 1375) and 2 mg NAD (Sigma D 5755)

Succinic acid dehydrogenase 0 1 ml of 0 6 N disodium succinate (Fluka AG, 14170 (A51172)

Glucose 6 phosphate dehydrogenase 0 1 ml of dipotassium glucose 6 phosphate (Sigma G7375) and 2 mg NADP (Sigma, T9002)

After the incubation the tissue sections are washed thoroughly in distilled water Contrast staining is not undertaken

Control sections are incubated without addition of the specific substrate, but otherwise under identical circumstances

RESULTS

The investigations may be divided in the following items, of which the first seven are concerned with the homogenate experiments

1 Absorption optimum for chloroform dissolved formazan
2 Stability of the solutions
3 Measurement of produced formazan on addition of an enzyme preparation containing G–6–DH
4 Measurement of homogenate activities of LDH, SDH and G–6–DH at increasing incubation periods
5 Measurement of produced amount of formazan at fixed incubation period, but addition of increasing amounts of homogenate
6 Reproducibility of the results
7 Influence of the pH values on the activities of G–6–DH in the enzyme preparation and in homogenates

8 Comparison of the results achieved by homogenate determination and by the histochemical method

1 *Absorption Optimum for Chloroform Dissolved Formazan*

The reducing agent sodium dithionite (Merck 6507) was added to the stock solution used for histochemical demonstration of dehydrogenases This caused formazan granules to precipitate as a coarse grained deposit The suspension was filtered, and the deposit dissolved in chloroform

An absorption curve was plotted on a Beckmann spectrophotometer with a variable wave range As shown in Fig 1, there is a plateau-formed optimum at about 540 nm The experiment thus showed the measuring value employed (540nm) to be suitable Further, plotting of an absorption curve will disclose impurities if present in the tetrazolium salt (5) (13)

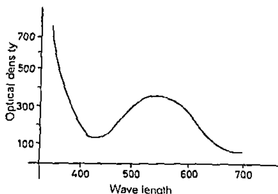


Fig 1 Absorption curve for formazan Solvent chloroform In the present study all the extinction measurements were carried out at wavelength 540 nm which corresponds to the absorption maximum

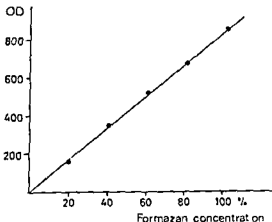


Fig 2 Relation between extinction and formazan concentration in the solution

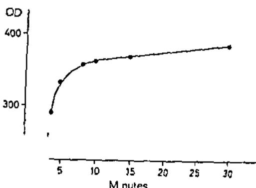


Fig 3 Activity in a solution formed of an enzyme preparation containing G 6 DH assessed by extinction of the produced amount of formazan (Note that the abscissa is elevated and accordingly does not intersect the ordinate at zero)

2 *Stability of the Solutions*

The stability of the solution was tested by repeated measurements of the extinction at a few days' intervals The chloroform dissolved formazan showed pronounced stability if the solution was stored under water If left standing in open air evaporation of the chloroform would cause a rapidly rising extinction of the solution

Next the extinction of the solution was studied on addition of increasing amounts of formazan The extinction was found to be directly proportional to the concentration of formazan in the solution As seen in Fig 2, the graph constituted a straight line passing through the zero point

3 *Measurement of Produced Formazan per Unit of Time after Addition of an Enzyme-Preparation Containing G-6-DH*

In these experiments an enzyme preparation containing G-6-DH was used (*Sigma*) Besides G-6-DH there was a *sufficient*

amount of the enzyme NADPH–Tr in the preparation, by which the colourless tetra zoliumsalts were reduced to the blue stained formazans

2 mg of G–6–DH preparation was added to 3 ml of phosphate buffer on pH 7 4 Of this solution, 0 4 ml was transferred to series of test tubes each containing 1 ml of incubation medium The reaction was interrupted after varying incubation periods, and the extinction was measured The results are seen in Fig 3 The enzyme activity had been high, with resulting high extinction values By prolonging the rectilinear part of the curve to the left until intersection with the ordinate, a high extinction is obtained, corresponding to the time 0 This is presumably due to a high initial rate of the enzyme reaction (1), (3), and the likely direction of the curve course is marked by the broken line

4 *Measurement of Homogenate Activities of LDH, SDH, and G–6–DH at Increasing Incubation Periods*

Parallel experiments were carried out under identical experimental circumstances The curves therefore gave an impression of the proportional activities of the individual enzymes in the same tissue mass All three graphs are rectilinear (Fig 4) A relatively

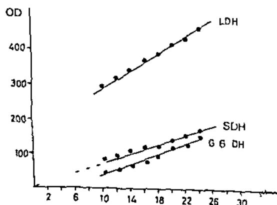


Fig 4 Homogenate activities of LDH, SDH and G 6-DH The curve illustrates the mutual intensities of the enzyme activities in the same tissue mass The broken line indicates the expected course of the SDH activity curve

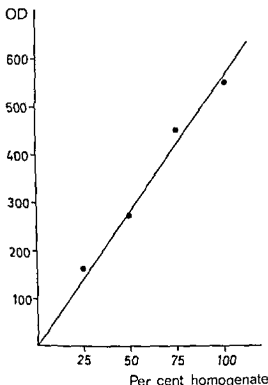


Fig 5 Measurement of the produced amount of formazan at fixed incubation period (10 minutes), but addition of increasing amounts of homogenate

low enzyme activity gives an optimal curve, i e a straight line traversing the zero point, as for instance for SDH At the very high extinctions, the sensitivity of the photometer is reduced, and the elution of the dyestuff may be incomplete Both factors explain why the relatively low extinctions give the best results

5 *Measurement of the Produced Amount of Formazan at Fixed Incubation Period, but Addition of Increasing Amounts of Homogenate*

The experiment showed whether the formazan production is proportional to the enzyme concentration, alternatively the homogenate concentration Fig 5 shows the curve which runs a rectilinear course through the zero point The curve corresponds, as might be expected, to that in Fig 2

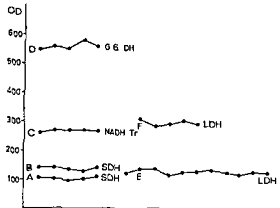


Fig 6 Diagram showing the reproducibility of the reactions A B, C, D, E, and F represent different tissue samples The repeated measurements have been set out in horizontally oriented columns connected by solid lines

6 *Reproducibility of the Results*

Equal amounts of homogenate and incubation medium were transferred to a series of test tubes Equal incubation periods gave equal results, i e good reproducibility, Fig 6 The values connected by horizontal lines indicate results of parallel experimental series

7 *Influence of pH on the Activities of G-6-DH in the Enzyme-Preparation and in Homogenates*

Varying quantities of hydrochloric acid were transferred to a series of test tubes containing incubation medium and homogenate The incubation then took place at different pH values The same incubation period (20 minutes) was employed for all the samples Fig 7 A + B illustrates the results in diagrammatic form Fig 7 A shows the activity of the enzyme preparation (G-6-DH) The blanks have been included in the graph, to show their uniform extinctions The activity curve itself (with the blank values subtracted) runs a rectilinear course towards a section on the abscissa corresponding to pH 5 5-6 0, i e the activity is measured at zero at this pH value Fig 7 B shows the homogenate activity of G-6-DH Note the greatly increasing blank values at rising pH

After subtraction of the blank values the curve runs in the direction of the same pH range as in Fig 7 A

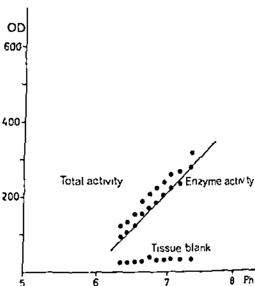


Fig 7 A Influence of pH on the enzyme activity The formazan production measured after addition of enzyme preparation containing G 6 DH to the incubation medium Measurements made at varying pH values but identical incubation periods

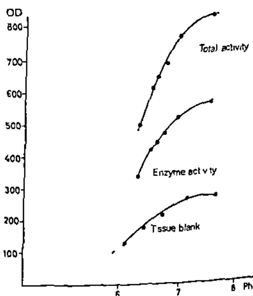


Fig 7 B Same experimental set up as in Fig 7 A, but with homogenate instead of the enzyme preparation

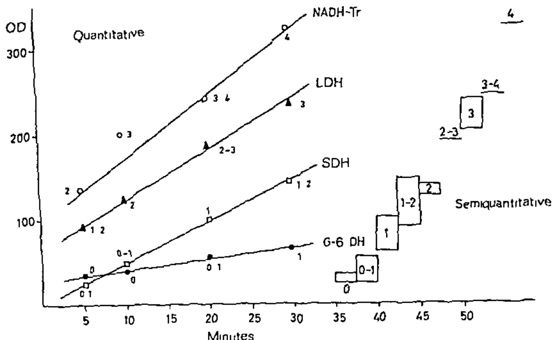


Fig 8 Comparative studies of the quantitative (colorimetric) and semiquantitative (histochemical) estimations Symbols ○, △, □, and ● indicate the colorimetrically measured values for NADH Tr, LDH, SDH, and G 6 DH, respectively The figures indicate the corresponding semiquantitative assessments To the right, rectangles indicate the variability of observations for the semiquantitative assessments By projecting the lower and upper limits of the rectangles on the ordinate, the corresponding extinctions can be read

8 *Comparison of the Results Achieved by Homogenate Determinations and by the Histochemical Method*

Tissue sections of rat liver were examined for activity of NADH-Tr, SDH, LDH, and G-6-DH by means of the previously described histochemical methods The samples were subjected to a semiquantitative assessment on the basis of the following divisions

0 absent reaction in the tissue samples
1 weak reaction only just suggesting the tissue structure
2 weak, but definitely positive reaction
3 strong, distinct reaction without excess staining
4 comprises all the samples with indistinct structures owing to excess staining caused by the high enzyme activity

The areas of the tissue sections were calculated on the basis of the semiquantitative assessment The outlines of the preparations were projected on a screen in ten times magnification, and the area was drawn on paper, from where it was cut out and weighed This procedure gives a relative measure of the amount of tissue, without, however, affording a possibility of taking into account variations in thickness of the sections

After the area measurement, the tissue sections were scraped off the slides with a razor blade The accumulated tissue amounts were minced by shaking in distilled water, after which the dyestuff could be eluted with chloroform and the extinction of the chloroform solution be measured The method is thus identical with that employed in the homogenate experiments

Fig 8 illustrates graphically the activities of NADH-Tr, LDH, SDH, and G-6-DH, estimated by a semiquantitative assessment of the tissue sections and by colorimetric meas-

urements of the chloroform dissolved formazan from the homogenized tissue sections The results achieved by the two methods were fairly alike (see also legend to Fig 8)

DISCUSSION

The choice of the ditetrazolium salt N-BT in all the experiments was due to a desire to create as uniform experimental conditions as possible allowing comparative studies on the semiquantitative, microscopical assessments and on the experiments with homogenization of the tissue material This involved use of the ditetrazolium salt nitro blue tetrazolium (N-BT) both in the homogenate experiments and in those with tissue sections, even though many workers claim that ditetrazolium salts are unsuitable for quantitative estimations (4), (7), (12), (14), (18)

It would however, be illusory to use N-BT for the tissue sections and a monotetrazolium salt for the homogenates, and then try to compare the results achieved with the two different indicators

The author chose N-BT for all the experiments owing to the extensive use of this indicator in histochemical demonstrations of dehydrogenase activity

To day the ditetrazolium salts are regarded as the most useful indicators in the histochemical staining methods for demonstration of dehydrogenase (9) (10) (20) The formazans produced display little diffusion presumably owing to a high affinity to protein This property is the reason why the substances are claimed to be fairly useless for quantitative analyses because the produced formazans are difficult to wash out

The present investigation showed however that the technical difficulties can be overcome, and in the literature available we also often find publications describing colorimetric measurements carried out on eluted formazans (1) (20)

Below, three factors will be discussed which created difficulties with regard to establishing the technique employed in the present study *1* Elution of the dyestuff *2* Interruption of the enzyme reaction *3* The "nothing dehydrogenase reaction"

1 Elution of the Dyestuff

After testing of a great number of solvents including acetone benzine, ether chloroform and trichlorethylene, chloroform was found to be the most useful

In the first experimental series chloroform was added to the incubation medium and the mixture was shaken vigorously The result was not satisfactory however, a stained deposit often persisting which could not be dissolved despite prolonged treatment After chloroform had been added to the homogenate this tended to form big intensely stained clots from which the formazan could not be eluted effectively If distilled water was added to the incubation medium prior to the addition of chloroform the deposit changed in character After gentle shaking the deposit grew finely granular and subsequent addition of chloroform effected elution of the total amount of dyestuff After centrifugation the now totally colourless deposit accumulated within the borderland between water and chloroform That the dyestuff had become totally eluted could be established by microscopy of the deposit or by additional chloroform treatment of the deposit during which persistent dyestuff would stain the chloroform

The added distilled water not only contributed towards effective elution by chloroform but also had an influence by settling as a protective layer on the stained chloroform solution thus preventing evaporation

2 Interruption of the Reaction

It is stated in several publications that the enzyme activity is blocked on addition of the solvent e g acetone The author made a series of experiments which showed that enzyme activity persisted in spite of addition of acetone to the incubation medium with a resulting rising extinction on standing Interruption of the reaction by heating the incubation medium was impossible because the

temperature rise resulted in an excessive increase of the enzyme activity before the temperature reached such a high level that the reaction ceased To block the enzyme reaction by altering the pH in the alkaline direction was not expedient because this might effect a non enzymatic reduction of the tetrazolium salt On the other hand change of pH in the acid direction will interrupt the enzyme activity with no undesirable side effects The employed addition of hydrochloric acid lowered the pH to under 2 This caused complete blocking of the enzyme reduction

3 The Concept of Nothing Dehydrogenase Reaction

It is employed to indicate presence of a non enzymatic reduction of the tetrazolium salt often seen on demonstrating dehydrogenase activity in tissue sections (2) (8) (11) (13) (15) (16) (19)

It is a commonly held view that this undesired reduction is caused by the SH groups present in the tissues which reduce the co enzyme with a resulting reduction of the tetrazolium salt In agreement with this the phenomenon is only seen in association with activity of the co-enzyme dependent dehydrogenases

Fig 7 B gives an example of the nothing dehydrogenase reaction In the experiment *outlined* in *Fig* 7 A the enzyme preparation containing G–6–DH was added to the incubation medium In Fig 7 B the enzyme was *also found* in the tissue homogenate in which SH groups were present too The result is seen in the blank values In Fig 7 B the non enzymatic reduction is seen to increase considerably at rising pH values It is therefore recommendable to carry out the experim

the non-enzymatic reduction (2)

The reactions in the homogenate experiments being identical with those employed in the histochemical demonstration of dehydrogenase activity it is plainly seen in Fig 7 B how important it is to include tissue sections incubated without the specific substrate (control sections) In the presence of nothing dehydrogenase reaction the control sections will be stained This colouring must accordingly be subtracted from the final result

It is evident from Figs 3–7 that the formazan production may be regarded as an indication of the presence of enzyme activity the curves being in agreement with corresponding illustrations in the universally recognized text books on enzyme kinetics (3), (6)

Fig 3 illustrates an activity curve plotted by an enzyme-preparation containing G–6–DH means of Fig 4 shows curves for enzyme activities in tissue homogenates The curves correspond in principle to that in Fig 3 Fig 5 demonstrates that addition of increasing amounts of homogenate (alternatively enzyme) effects corresponding rises of the formazan production Fig 6 shows that the results are reproducible and Fig 7 illustrate the nothing dehydrogenase reaction and the dependence of the enzyme activity on the pH

Next an account will be given of the comparative studies of the results of colorimetric and histochemical methods

The two series of experiments were carried out as blinds and the results of the colorimetric measurements were unknown to the investigator when the semiquantitative assessments were performed As shown in Fig 8 the results of the two experimental series are in fair agreement By comparing Fig 4 and Fig 8 the curves were likewise found to be identical i e alike whether the experimental material consisted of tissue blocks homogenized (and incubated) or tissue sections incubated and thereafter washed in chloroform

It is seen in Fig 8 that one of the values on the NADH Tr s reaction curve (10 minutes of incubation) gave a higher extinction than might be expected A similar high value was however also found on the semiquantitative assessment of the tissue section This is evidence suggesting that the variation in

both parameters depends on an increased section thickness (as stated previously, the same tissue sections were used for the quantitative and the semiquantitative assessment) Semiquantitative assessments of tissue sections and quantitative measurements of homogenates thus gave concordant results

CONCLUSION

The conclusion may be drawn from the results of the present investigation that on measuring dehydrogenase activity by the tetrazolium salt method the amount produced of formazan may be taken as an indication of the enzyme activity, provided the blank value with a possible "nothing dehydrogenase-*reaction" has been subtracted Semiquantitative* assessments of tissue sections and quantitative measurements on homogenates give concordant results The methods are specific and the results reproducible

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The work was aided by grants from *Kobmand i Odense Johan og Hanne Weimann f Seedorffs Legat*, and *Daell Fonden*

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Acta path microbiol scand Section A, *80*, 557–565 1972

HISTOLOGICAL CHANGES IN HUMAN LIVER BIOPSIES FROM CHRONIC ALCOHOLICS

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In a material consisting of liver biopsies from 330 consecutive patients with chronic alcoholism (alcohol consumption of more than 50 g/day for a period of greater duration than a year) 60 (18 per cent) show cirrhosis or have given a suspicion of cirrhosis 56 of these in addition exhibit varying degrees of fatty change 212 biopsies (64 per cent) show fatty change without cirrhosis 23 (seven per cent) biopsies as the most important pathological finding exhibit varying degrees of iron storage in the liver cells and 27 biopsies (eight per cent) show normal liver tissue without iron content and without a single cell with fatty change The last eight biopsies show hepatitis or non specific reactive changes

While there are numerous investigations concerning which morphological changes are seen in liver biopsies from selected groups of alcoholics with fatty change and/or cirrhosis (1, 24, 20), only few reports give information of *which* changes are seen in consecutive materials of biopsies from chronic alcoholics (9)

The purpose of the present paper has been to describe the incidence and the degree of different histological qualities in a consecutive series of liver biopsies from chronic alcoholics from a Scandinavian metropolis.

MATERIAL AND METHODS

The material consists of 330 percutaneous liver biopsies from 330 consecutive patients with chronic alcoholism admitted to department II, Kommunehospitalet, during the period January 1968 to March 1971

The liver biopsies have been performed by the method of Menghini when there was a history of alcohol consumption of more than 50 g/day for a period of greater duration than a year All the patients in department II, and in cases of doubt their nearest relatives, have in the period of investigation been specifically questioned on this point by one of the authors (K N)

The tissue has been fixed in neutral formalin and imbedded in paraffin The biopsies are 1–1 4 mm thick and 1 5–4 5 cm long and are cut in serial sections (6 µm – 50 sections)

The assessment has been performed by one of the authors (P C) on haematoxylin and eosin and v Gieson Hansen stained sections Furthermore, additional sections from all biopsies stained for reticulin fibres (10), iron (16), and pyroninophil substance (2) have been available

The occurrence of the following qualities has been semiquantitively registered (0–3) Mallory bodies (5), alcoholic hepatitis (3) focal necroses (liver cell necro[illegible]
he[illegible]
hi[illegible]
tracellular bile thrombi), lipogranulomas (4), parenchymal inflammation, bile duct proliferation and inflammation of the portal tracts, as well as iron deposits in liver cells, Kupffer cells and connective tissue Further the degree of fatty change and fibrosis, as well as whether the normal lobular

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architecture is preserved or cirrhosis has developed have been registered

Fatty change is quantified in the following manner

- 0 the biopsies contain no fat vacuoles
- \+ the biopsies contain fatty vacuoles, but on average in less than one third of the cells
- \+ + the biopsies contain fatty vacuoles in one third or more of the cells but in less than two thirds of the cells
- \+ + + the biopsies contain fatty vacuoles in two third or more of the cells.

In addition an estimation of the biopsy as an entity has been performed with a classification of the material according to chief histological diagnoses

RESULTS

Table 1 shows the distribution according to chief histological diagnosis of the 330 biopsies comprising the material

TABLE 1 *Distribution of the 330 Biopsies of the Material According to Chief Histological Diagnosis*

Chief histological diagnosis	Number of biopsies
Cirrhosis	54
Obs cirrhosis	6
Fatty liver	212
Haemosiderosis	23
Viral hepatitis	3
Chronic aggressive hepatitis	2
Liver with non specific changes	2
Liver without pathological changes	27
Unsuitable biopsy	1
	330

Cirrhosis

54 of the biopsies (16 per cent) fulfill the histological criteria for cirrhosis (nodular regeneration and fibrosis) In 21 cases the cirrhosis is of regular type with regeneration noduli which are alike and of the size of or smaller than lobuli, while the remainder are of irregular type with regeneration noduli of most variable size

A summary of the *parenchymal changes* in the 54 biopsies with cirrhosis is given in Table 2

TABLE 2 *Number of Biopsies with the Following Parenchymal Changes from 54 Patients with Cirrhosis*

Parenchymal changes		
Fatty change	slight	15
	moderate	31
	severe	4
Focal necroses		54
Lipogranulomas		32
Mallory bodies		23
Alcoholic hepatitis		18
Kupffer cell proliferation		54
Inflammation		36
Cholestasis		8
Iron content		18

50 of the 54 biopsies with cirrhosis show some degree of fatty change and in the majority of cases this is moderate The four biopsies with cirrhosis and without fatty change all exhibit an irregular cirrhosis but apart from this there is no difference in the degree of fatty change or the other registered qualities in the two types of cirrhosis In only one of the cases many focal necroses are found In all cases they are distributed diffusely throughout the parenchyma The number of lipogranulomas is in all small and it is in all cases type I and/or type II with a predominantly diffuse distribution

Mallory bodies have been found in 23 biopsies and all biopsies with Mallory bodies show some degree of fatty change In none of the 23 biopsies have many Mallory bodies been demonstrated and 13 only contain a slight number and ten a moderate Mallory bodies may be found in all areas of the parenchyma but are however most frequent in the marginal areas of the nodulus

18 (78 per cent) of the 23 biopsies with Mallory bodies in addition show changes as in alcoholic hepatitis with necroses of liver cells containing Mallory bodies and infiltration with neutrophils in the surroundings

There is a slight degree of Kupffer cell proliferation in all the biopsies with cirrhosis 36 biopsies in addition contain a few inflammatory cells in the parenchyma Plasma cells

TABLE 3 *Number of Biopsies with the Following Portal Changes from 54 Patients with Cirrhosis*

Portal changes		
	slight	3
Fibrosis	moderate	40
	severe	11
Bile duct proliferation		54
Inflammation		54

have not been demonstrated in the parenchyma Lymphocytes are seen in 21, and granulocytes in 21 biopsies

Eight biopsies exhibit slight cholestasis and 18 biopsies exhibit a slight to moderat iron content in the liver cells

A summary of the *changes in the connective tissue* is given in Table 3

In 11 cases a pronounced fibrosis is seen, while 43 only present a slight or moderate increase in connective tissue

All 54 biopsies with cirrhosis exhibit bile duct proliferation The proliferation is severe in five cases, while the remaining 49 only exhibit slight to moderate proliferation

The inflammatory reaction in the connective tissue is pronounced in 11 biopsies, while 27 and 16 cases exhibit respectively slight and moderate cellular infiltration Lymphocytes and histiocytes have been demonstrated in all cases neutrophils in 40, and plasma cells and eosinophils in respectively 13 and 10 biopsies

One of the biopsies with cirrhosis but no fatty change shows bile duct with atypical epithelium (22) and in addition a dense infiltration of the connective tissue in the septa with lymphocytes and plasma cells, and one of the biopsies with cirrhosis and fatty change in addition shows hepatoma

Suspicion of Cirrhosis

6 biopsies (2 per cent) have given a suspicion of cirrhosis but incontrovertible nodular regeneration has not been recognized All six cases show some degree of fatty change and exhibit a slight to moderate iron content in the liver cells Three cases exhibit a few Mallory bodies and two of these in addition alcoholic hepatitis

Fatty Liver (Without Cirrhosis)

Somewhat more than half (64 per cent) of the biopsies show fatty change without cirrhosis

TABLE 4 *Number of Biopsies with the Following Parenchymal Changes from 212 Patients with Fatty Change without Cirrhosis*

Parenchymal changes		
	slight	106
Fatty change	moderate	77
	severe	29
Focal necroses		193
Lipogranulomas		86
Mallory bodies		13
Alcoholic hepatitis		9
Kupffer cell proliferation		209
Inflammation		27
Cholestasis		8
Iron content		122

A summary of *the parenchymal changes* in the 212 biopsies with fatty change without cirrhosis is given in Table 4 One half of the biopsies in this group exhibit slight fatty change Included as slight fatty change in this material have also been biopsies which only contain a few lipid droplets in each lobule Somewhat more than ⅓ of the biopsies exhibit moderate fatty change, and only 29 (13 per cent) show severe fatty change

A few focal necroses, distribuated diffusely throughout the parenchyma, are found in most biopsies and lipogranulomas are found in 86 biopsies (41 per cent) In 83 cases the lipogranulomas exclusively belong to type 1 and 2, while three biopsies in addition contain lipogranulomas type 3

Thirteen biopsies (six per cent) contain Mallory bodies The majority (9 biopsies) contain only a few Mallory bodies, while four contain a moderate number In biopsies with fatty change without cirrhosis Mallory bodies are most often found centrilobularly

Alcoholic hepatitis is demonstrated in most biopsies with Mallory bodies (70 per cent)

All biopsies with the exception of three exhibit some degree of Kupffer cell proliferation, and the proliferation is in all cases slight.

The degree of inflammation in all 27 biopsies with inflammatory cells is slight Plasma cells have not been demonstrated in the parenchyma Lymphocytes are seen in 17 biopsies, neutrophils in 13, and eosinophils in four

Eight biopsies exhibit slight cholestasis All biopsies contain lipofuscin and about half (58 per cent) iron in liver cells

TABLE 5 *Number of Biopsies with the Following Portal Changes from 212 Patients with Fatty Change without Cirrhosis*

Portal changes	
Fibroses	57
Bile duct proliferation	23
Inflammation	123

A summary of the changes in the portal tracts is given in Table 5

In two cases a moderate diffuse fibrosis is seen, while 55 only present a slight increase in connective tissue

In 19 of the cases with bile duct proliferation it is diffusely distributed in the portal tissue, whereas in four cases a bile duct proliferation localized to the peripheral part of the portal area's connective tissue has been demonstrated in very close relationship to the limiting membrane ("marginal bile duct proliferation") The same four biopsies exhibit a slight degree of cholestasis The degree of bile duct proliferation is in all 23 cases slight

Somewhat more than half of the 212 biopsies (58 per cent) contain inflammatory cells in the portal connective tissue In 113 cases the inflammatory infiltrate is slight, in 8 moderate and only two biopsies exhibit severe portal inflammation Lymphocytes and histiocytes have been demonstrated in all 123 cases, neutrophils in 43 plasma cells in six and eosinophils in five biopsies

Haemosiderosis

23 biopsies (seven per cent) show some degree of iron content in the liver cells without simultaneous fatty change and/or cirrhosis As seen in Table 6 the iron content is in the majority of the biopsies moderate, and only one contains larger amounts of iron

TABLE 6 *Number of Biopsies with the Following Changes from 23 Patients with Haemosiderosis without Fatty Change or Cirrhosis*

	slight	7
Iron content	moderate	15
	severe	1
Focal necroses		16
Kupffer cell proliferation		21
Parenchymal inflammation		0
Portal inflammation		1
Portal diffuse fibrosis		1

Focal necroses are found in somewhat more than two thirds of the biopsies In none of the cases are many focal necroses found, and they are in all cases distributed diffusely throughout the lobules

In 21 cases there is a slight Kupffer cell proliferation Lymphocytes, neutrophils or plasma cells have in no case been demonstrated in the parenchyma, and only one biopsy, the one with a severe degree of iron content, exhibits slight portal inflammation with a few lymphocytes, neutrophils and histiocytes in the portal tracts This same biopsy as the only one in this group exhibits slight diffuse portal fibrosis

Lipofuscin is seen in all 23 biopsies, in a slight degree in 14, in a moderate in 7, and in a severe degree in two

"Viral" Hepatitis

Three biopsies show changes as in acute hepatitis with 1) focal liver cell necroses, 2) condensation of reticulum fibres 3) focal Kupffer cell proliferation 4) focal infiltration with lymphocytes 5) ballooning of liver cells 6) phleomorphism of nuclei and 7) acidophilic bodies The changes are mainly centrilobular

In addition all three biopsies show some degree of portal inflammation with lymphocytes, histiocytes and a few plasma cells

None of the biopsies contain fat, but in all three cases the liver cells contain small

amounts of iron Iron has not been demonstrated in Kupffer cells or in portal macrophages

Chronic Aggressive Hepatitis

Two biopsies exhibit changes in the portal tracts as in chronic aggressive hepatitis with a slight degree of diffuse fibrosis and an inflammatory reaction with lymphocytes, histiocytes and many plasma cells, and in addition a moderate number of piecemeal necroses with many plasma cells Further a slight diffuse bile duct proliferation is found in the portal tracts

In the parenchyma a few focal necroses, scattered plasma cells, and slight Kupffer cell proliferation are found, but no superimposed acute 'viral' hepatitis None of the biopsies exhibit fatty change, but in one of them the liver cells contain small amounts of iron

Non Specific Reactive Hepatitis

In two biopsies as the only finding single focal necroses in the parenchyma with a diffuse distribution are found In addition a slight degree of Kupffer cell proliferation and in one of the biopsies a slight portal fibrosis are found None of the biopsies contain iron or fat

Liver without Pathological Changes

27 biopsies (eight per cent of the material) show normal liver tissue without iron content and without a single cell with fatty change

Unsuitable Biopsy

Only one biopsy (0 3 per cent) contained insufficient liver tissue for a morphological evaluation

DISCUSSION

Cirrhosis in Chronic Alcoholics

Frequency For over a century clinicians and pathologists have recognized a relationship between over indulgence in alcohol and the occurrence of cirrhosis. Autopsy investigations have shown a distinct correlation between alcohol intake and death from cirrhosis not only from country to country but also in the same area During the prohibition period in the U S A (1916–32) deaths from cirrhosis were halved and during the wine rationing in Paris from 1942–46 the number of deaths from cirrhosis fell from 35 to 6 per 100 000 (17) In both areas the number of deaths from cirrhosis has again risen in proportion to rising consumption of alcohol (11)

Several investigations have shown that only about 10 per cent of chronic alcoholics have cirrhosis *Viel et al* (26) in a study of 777 men between 10 and 69 years of age who had died of a violent cause found that 45 per cent of the persons had been heavy drinkers or alcoholics Nine per cent of these alcoholics showed cirrhosis of the liver

Lelbach (14) examined liver biopsies from 320 alcohol addicts obtained from a material of 526 alcoholics and found that in 12 per cent of the cases cirrhosis was present

The quoted results are thus either from autopsy materials or from non consecutive materials and do perhaps not give the true incidence of cirrhosis in chronic alcoholics

The present material which comprises biopsies from all known alcoholics who have been admitted to II department, Kommunehospitalet during the period of investigation has been consecutive, but of course selected as it only comprises alcoholics who have been admitted to hospital 54 (16 per cent) of the 330 chronic alcoholics in the material have cirrhosis and an additional six have changes in their biopsy giving suspicion of cirrhosis

It is noteworthy that in the present material which comprises consecutive alcoholics there is a higher incidence of cirrhosis than in the quoted non consecutive materials An investigation of the significance of the time factor is in preparation A considerable part of the explanation of this high incidence no doubt lies in the extensive use of serial sections and staining for reticulum fibres which experience *shows to confirm* the diagnosis, even when the changes are minimal (23)

Devenyi et al (9) in a consecutive material consisting of 100 chronic alcoholics found, that 65 showed fatty infiltration, one ad-

vanced portal cirrhosis, and 34 were normal It is interesting, that there in this material which consists of patients with a duration of excessive drinking between 5 and 25 years is only one case of cirrhosis Further morphological information has not been given in this work, but the expression "advanced portal cirrhosis" makes it probable, that several less advanced cases of cirrhosis are included in the group with fatty infiltration

Morphological features in cirrhosis from chronic alcoholics

Type of cirrhosis Barely one half (39 per cent) of the 54 patients with cirrhosis have a classical "alcoholic" or "nutritional" cirrhosis with identical small nodules of regeneration This finding is in accordance with the results reported by *Popper et al* (18)

Popper et al (18) in a material of autopsy specimens from 309 patients with cirrhosis found that in the patients with established histories of alcoholism about half were designated as portal or regular cirrhosis, whereas the other half were of the irregular or postnecrotic type On this basis, as well as the presense of several stages in the same specimen, an evolution from regular cirrhosis with fatty change via necrosis, collapse, and regeneration to irregular cirrhosis as a terminal pathway is postulated It is the opinion of *Popper et al* (18) that the increased frequency of irregular cirrhosis in recent years may reflect larger survival as a result of better management

Fatty change Incidence and degree of fatty change in alcoholic cirrhosis varies depending on the kind of material Both *Baggenstoss & Stauffer* (1) and *Rubin et al* (24) quite frequently find alcoholic cirrhosis without fatty change Both materials are autopsy materials and it is probable that the patients have previously had fatty livers that have disappeared terminally following treatment and stay in hospital *Porta et al* (21) thus state that a fatty liver may disappear inside a few weaks prior to death Corresponding with this *Summerskill et al* (25) find fatty change in 75 per cent of an autopsy material and in 95 per cent of a biopsy material

In the present material 50 (93 per cent) show some degree of fatty change The four biopsies with cirrhosis without fatty change al exhibit an irregular cirrhosis

Mallory bodies and alcoholic hepatitis In the part of the present material that comprises alcoholics with cirrhosis and fatty change Mallory bodies have been demonstrated in 46 per cent *Rubin et al* (24) in an autopsy material of non consecutive cases of cirrhosis found that "among alcoholics" more than a third exhibited Mallory bodies

Baggenstoss & Stauffer (1) and later *Popper & Szanto* (20) in non-consecutive autopsy materials of alcoholics with cirrhosis demonstrated Mallory bodies in 72 and 93 per cent respectively

Christoffersen (6) demonstrated Mallory bodies in 45 (38 per cent) out of 120 consecutive biopsies with cirrhosis and fatty change Nearly all the patients with cirrhosis and fatty change in the last mentioned paper were clinically suspected of being chronic alcoholics, and 84 per cent confirmed the diagnosis themselves (3) The figures for alcohol consumption are absolute minimum values since the information has been taken from case records, and only part of the patients have been specifically questioned on this point

The incidence of Mallory bodies in alcoholics with cirrhosis and fatty change thus appears to be 38–47 per cent in consecutive materials

The present material contains only four biopsies with cirrhosis without fattychange In none of these Mallory bodies are demonstrated

Alcoholic hepatitis is found in 78 per cent of the biopsis with cirrhosis and Mallory bodies

Activity In liver tissue with cirrhosis and fatty change it is possible to speak about two morphologically different kinds of activity, namely alcoholic hepatitis and the other kinds of liver-cell necroses with mesenchymal reaction (8) Previous investigations (7) have made it probable, that alcohol not only induces alcoholic hepatitis but also the other

kinds of necroses that may be demonstrated in liver tissue with cirrhosis from alcoholics. The present material which is derived from alcoholics with an abuse still taking place tends to confirm this assumption, as some degree of activity has been demonstrated in all biopsies with cirrhosis.

Fatty Change in Chronic Alcoholics

Frequency. Somewhat more than three quarters (81 per cent) of the biopsies show fatty change (ranging from single vacuoles per lobule to vacuoles in nearly all liver cells).

Leevy et al. (12) found that somewhat more than two thirds of the patients admitted for delirium tremens to Jersey City Medical Center exhibited a significant increase in liver fats. *Lelbach* (14) examined liver biopsies from 320 non-consecutive alcoholics and found fatty change in approximately three quarters of his material.

Viel et al. (26) in a non-hospitalized material found fatty change in a little more than half of the alcoholics.

56 of the 268 biopsies with fatty change exhibit cirrhosis or give suspicion of cirrhosis and have been discussed above. The following applies to the 212 biopsies with fatty change without cirrhosis.

Frequency of fatty liver without cirrhosis. In the present material fatty change without cirrhosis has been demonstrated in 64 per cent of the biopsies.

Lelbach (14) examined liver biopsies from 320 alcoholics and found that in approximately 37 per cent there was evidence of "uncomplicated, more or less marked fatty liver", whereas 29 per cent showed fatty liver and in addition an inflammatory mesenchymal reaction. *Lelbach* (14) thus in all finds fatty change without cirrhosis in 66 per cent of his material.

It is noteworthy that the incidence of fatty change is nearly identical in the two rather different materials mentioned. Lelbach's material comprises biopsies from 320 out of a group of 526 chronic alcoholics, while our material is consecutive. Lelbach does not more closely explain whether the 206 not having biopsies taken are patients with slighter or more severe clinical and biochemical symptoms, but it is probable that they were alcoholics with slight or no symptoms and therefore less motivated to have a biopsy performed. A greater incidence was therefore to be expected in Lelbach's material than in ours. When this is not the case, the explanation is probably, that we as liver with slight fatty change also have included biopsies only containing a few lipid droplets per lobule.

An other probable explanation is the very long period of abstinens, before the biopsy was performed in Lelbach's material (77±54 days).

Morphological Features in Livers from Chronic Alcoholics with Fatty Change without Cirrhosis

Degree of fatty change. In the slightest degrees of fatty change only a few and isolated liver cells are found containing lipid droplets. In moderate fatty change the infiltration is usually diffuse in the lobules but may be either predominantly centrilobular or periportal.

When the lipid content of the liver rises over the normal 5 g per 100 g liver tissue to 20–30 g the steatosis become uniform throughout the liver and the pathologist speaks of a "swiss cheese appearance" (19). The hepatocytes are uniformly filled with large fat droplets and eventually fat drops of several neighbouring cells coalesce to a larger fatty cyst which now lies extracellularly, producing an inflammatory reaction (19).

Mallory bodies and alcoholic hepatitis. There seems to be an intimate connection between Mallory bodies and fatty change, and it is probable that Mallory bodies only arise when there is fatty change, even though the two phenomena are not seen in the same cell.

On the other hand the considerable degree of positive correlation between the frequency of Mallory bodies and degree of fatty change (7) speaks in favour of a temporal and aetiologic connection.

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The frequency of Mallory bodies in fatty liver without cirrhosis varies according to the criteria used in the selection of the material *Popper and Szanto* (20) in a material selected on both morphological and clinical criteria found Mallory bodies in 100 *per cent* of "large fatty livers" from alcoholics In the present material Mallory bodies have been demonstrated in 13 (six *per cent*) of 212 biopsies

It is noteworthy that Mallory bodies in the material of biopsies with fatty change from alcoholics are found with the same incidence as in materials of biopsies with fatty change without information regarding the alcohol consumption (6) This indicates that the patients with fatty change of the liver in the referred paper (6) to a large extent comprise alcoholics

It thus appears that the incidence of Mallory bodies in alcoholics with fatty change but no cirrhosis is about six per cent

Alcoholic hepatitis is found in 70 per cent of the biopsies with fatty change without cirrhosis but with Mallory bodies

Activity Just as in liver tissue with cirrhosis and fatty change it is possible to speak about two morphological different kinds of activity in fatty liver without cirrhosis namely alcoholic hepatitis and the other kinds of liver cell necroses with mesenchymal reaction (7) The fact, that some degree of activity is found in nearly all biopsies with fatty change without cirrhosis (99 per cent) from a material of alcoholics with an abuse still taking place, tends to confirm the assumption, that alcohol not only induces alcoholic hepatitis but also the other kinds of necroses

Iron Content in Liver Biopsies from Chronic Alcoholics

It is usually assumed that chronic alcoholism and increased iron storage are associated (13)

Increased content of iron in alcoholics is seen both when the liver tissue otherwise is normal, exhibits fatty change and/or cirrhosis In the present consecutive material consisting of 330 alcoholics 173 (52 per cent) have increased amount of iron in the liver tissue In 23 cases the liver was otherwise normal, in 122 cases it exhibited varying degrees of fatty change, and 24 cases exhibit cirrhosis or gave suspension of cirrhosis The last four patients had acute hepatitis (3) and chronic aggressive hepatitis (1)

Patients addicted to alcohol have many reasons for developing an iron overload The high iron content of some alcoholic drinks (15) may contribute to increased iron stores Alcohol has also been reported to increase the absorption of ferric iron (15) and steatosis was reported to be associated with increased iron absorption in man

In actual practice, there appears to be considerable overlap (13) and at present it is extremely difficult to determine the significance and mechanism of iron overload in an alcoholic subject

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This work was supported by grants from *Statens Lægevidenskabelige Forskningsråd*

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ON FACTORS RELATED TO SPONTANEOUS HEALING OF RUPTURED INTRACRANIAL ANEURYSMS

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Ninety four intracranial aneurysms were examined with the aim of finding what spontaneous organizing processes may lead to strengthening of the aneurysmal wall The aneurysms were examined with a dissecting microscope, by microradiography and histopathologically The following types of changes affording protection against recurrence of subarachnoid haemorrhage were considered a) innervation in relation to spasm close to the neck of the aneurysm, b) thrombosis of the aneurysm, c) reparative processes at the site of rupture, d) development of whole new walls, e) organizing processes related to fatty atheromatoses and calcifications, f) possible regeneration of elastic and muscular tissue, and g) formation of new blood vessels in the walls of the aneurysms No real regeneration of elastin or medial layer seems possible, however, pre-existing clumping fragmented elastica may be seen During development of either a membrane or an atheroma leading to calcification, there is still a danger of rebleeding until the process is complete in relation to the aneurysmal wall, i e, until a complete tight new sac has formed or a yellow thrombus completely filling the aneurysmal sac has developed or a complete calcification of the entire aneurysmal wall has taken place

Some intracranial aneurysms never bleed and others form thromboses spontaneously and develop permanent new walls after the first subarachnoid haemorrhage Aneurysms taken at autopsy have been investigated by various methods, with the aim of obtaining information concerning protective processes within and around the aneurysmal sac Special attention has been paid to factors preventing recurrent haemorrhages, such as reparative processes in the aneurysmal wall The following factors have been studied 1) innervation in relation to spasm, 2) thrombosis of the aneurysm, 3) reparative processes at the site of rupture of single-walled aneurysms, 4) organizing processes, such as fibrosis of atheromas and calcifications, in the aneurysmal wall, 5) possible regeneration of elastic or muscular tissue in connection with reparative processes, 6) development of several separate walls i e, "onion aneurysms", 7) neoformation of small vessels in the walls of the aneurysms

MATERIALS AND METHODS

Ninety four aneurysms taken at autopsy from 75 cases were investigated Eighteen patients had died ... ...morrhage ... had a ... one or

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more years before death Among the 57 patients who died from subarachnoid haemorrhage, 26 succumbed within 1 week, 12 within 2 weeks 9 within 3 weeks and 10 between 3 and 6 weeks after the last bleeding Twenty four patients had had more than one bleeding There were no significant sex differences in the series The mean age was 52 The largest diameter of the aneurysms varied between 4 mm and 3 cm.

The following methods of investigation were employed

1 All the aneurysms were examined under a dissecting microscope The vessels carrying the aneurysms were dissected out with the aneurysms and with strands of thickened arachnoid where these participated in posthaemorrhagic organizing processes

2 Microradiography of all the aneurysms was done by conventional methods (*Bostrom & Hassler* 1965, *Hassler* 1964) Parts of the specimens were embedded in methylmethacrylate Modified Practix (Philips) and Debyeflex (Rich Seifert) microradiography (tube PV 754100 with tungsten anode) apparatuses were used for the investigations The radiation was generated at 28 30 kv The photographic material used was Kodalith Ortho Type 3 film

3 Micro angiography was carried out in 10 specimens with Micropaque as dye The aneurysms for micro-angiography were rinsed with saline, filled with the dye through the basal vessels and then dissected out, the inner surface of the aneurysm was rinsed again with saline and finally the sac was micro radiographed

4 Sections of all aneurysms were stained with resorcin v Kossa van Gieson and Sudan III

RESULTS

Innervation of the Neck of the Aneurysm

Bunches of adventitious nerves reaching over the neck of the aneurysm were found in 6 cases In four of these the aneurysm had ruptured The nerve bunches were visible under the dissecting microscope and were seen in cross section in the histological preparations The nerves continued along the basal vessels

Thrombosis of the Aneurysms

In the present material there were 14 aneurysms in which only part of the sac was thrombosed, the mass resembling a small clot without evidence of any large amount of thrombocytes Twenty six aneurysms were wholly thrombosed In three of these cases there had been no verified bleeding Cross sections of the thrombosed aneurysms were primarily analysed with the dissecting microscope and some parts sliced for histopathological staining The aneurysms generally contained laminated masses with a "tigroid" appearance The lamination was frequently concentric Histopathological examination showed that the light parts of the thrombi consisted of blood platelets, phagocytes, fibrin and cholesterol In five instances there were clear signs of organization of the thrombus by collagen fibres and fibroblasts An inner membrane was present and was generally less well developed in the peripheral parts close to the true wall of the aneurysm than in its middle part (Fig 1) The connective tissue membrane was bordered with fat Four aneurysms were filled with a homogeneous yellow mass staining bright red with Sudan III Three of these had apparently never ruptured In the fourth aneurysm, the mass only occupied part of



Fig 1 Photograph of cross sections of aneurysms taken at autopsy Upper left Unruptured anterior communicating artery aneurysm from 68 year-old patient The sac is entirely filled with white thrombus masses Upper right Ruptured anterior communicating artery aneurysm from 45 year-old patient Middle and right part of aneurysm filled with white thrombus (arrow) In left part of aneurysm dark blood *coagulum* At the dome of this aneurysm a small calcification was seen in microradiography Lower part of figure Ruptured middle cerebral artery aneurysms The intra aneurysmal membrane formation is clearly visible in the aneurysm in the middle Natural size

Reparative Processes at the Site of Rupture of Single-Walled Aneurysms with or without Secondary Outpouchings

Eighty aneurysms which were saccular in shape had a single wall They were examined under a dissecting microscope Secondary outpouchings were present in 21 Remnants of secondary outpouchings were present at the site of rupture in 11 additional aneurysms In 5 cases in which bleeding had not been fatal, dense layers of fibrous tissue covering these outpouchings were seen in preparations stained with v Gieson stain

In one 42-year-old patient, a pea sized aneurysm of the right middle cerebral artery was visualized at angiography at the first episode of subarachnoid haemorrhage (Fig 2 a) At a second bleeding verified 10 years later, the aneurysm had enlarged to the size of a cherry (Fig 2 b) The patient died in consequence of this second bleeding Microradiographic study showed a minute calcification in the wall of the original pea sized aneurysm Adjacent to the calcification was a greatly enlarged thin walled sac By histopathological study, the aneurysmal wall was found to consist partly of a piece of thickened pia arachnoid (Fig 3) partly of a neoformed haematoma membrane



Fig 2 a Part of carotoid artery angiogram of ruptured peasized middle cerebral artery aneurysm (arrow) in 32 year old patient Natural size



Fig 2 b Part of cartoid artery angiogram of same patient where lesion is shown in Fig 2 a Now the ruptured aneurysm is cherry sized (arrow) and 10 years have past since the first angiography The pea sized original aneurysmal sac is hardly visible as a black spot behind the cherry sized sac Natural size

the lumen although extending from the neck to the dome of the sac, calcification of the latter was observed by micro-radiography Rupture had occurred at one side of the thrombus



Fig 3 Microphotograph of small vessels on lumen side of thickened pia arachnoid forming wall of middle cerebral artery aneurysm seen in Fig 2 b No proper internal elastic lamina or media layer present in aneurysmal wall Haematomatous mass visible in lower part of figure Resorcin fuchsin × 100



Fig 4 Microradiograph of non ruptured totally calcified anterior communicating artery aneurysm. Autopsy finding in 60 year old patient × 2

Fibrosis of Pre Atherosclerotic Fatty Plaques and Calcifications

The organization and incidence of fatty plaques and calcifications were investigated with stereomicroscopic, microradiographic and histopathological methods Fatty infiltrated plaques were found in the walls of 92 of the aneurysms They were present at the sites of outpouchings The lipid mass of



Fig 5 Microphotograph of part of ruptured anterior communicating artery aneurysm in 32 year old patient Large part of atheroma on lumen side of aneurysm almost detached from wall Sudan III × 47

plaques was occasionally covered by a thin layer of connective tissue In the walls of the empty outpouchings vestiges of fatty infiltrates were visible The fatty plaques were easy to separate from the walls In 20 aneurysms, a fatty plaque was organized by fibrosis into a thicker mass that was not separable from the wall Lymphocytes and phagocytes were present in these fibrotic masses Calcifications were present in the walls of 32 aneurysms mainly at the dome or neck of the aneurysms or as a girdle at their largest diameter Two aneurysms were completely calcified (Fig 4) Calcifications of notable size were only invested in thin connective tissue layers

Elastic and Muscular Tissue in the Aneurysmal Walls

Remnants of elastic or muscular tissue were seen to extend over the neck into the bulging parts of the wall of 12 aneurysms, six of which were unruptured In the aneurysm material the part of the mother vessel which surrounds the origin of the neck of the aneurysm elastic and muscular tissue generally was intact In four aneurysms with ruptures in the elastica atheromatous masses were situated in a fibrous layer between the adventitia and the elastic lamina in two aneurysms between the elastic lamina and the intima (Fig 5) The atheromatous plaques did not contain any muscle components There were no signs of regeneration of elastin or muscle cells in conjunction with the reparative processes in these ruptured aneurysms

Several Separate Walls, i e, "Onion Aneurysms"

Among the cases with a history of several bleedings 14 aneurysms with several walls were found These aneurysms were of considerable size Their diameters ranged from 1 5 to 3 cm Four aneurysms had 3 separate, almost equally thick walls of similar microscopic appearance Ten aneurysms had 2 separate walls, the outer wall being thicker



Fig 6 Photograph of internal carotid artery aneurysm Autopsy finding in 39 year-old patient Outer shell of aneurysm consists partly of pia arachnoid partly of haematoma membrane × 2

in two and the inner wall in eight In one patient, during a posthaemorrhagic interval of 4 days a new, extremely thin wall had formed around a blood clot adherent to the original aneurysmal wall In histopathological study the outer wall consisted of fibrin thin collagen bundles fibroblasts and macrophages The arachnoid membrane formed part of this new aneurysmal wall Among the 8 cases with such aneurysms the youngest was a 3 month old infant with an aneurysm of the right middle cerebral artery In another patient, an adult with an aneurysm of the left internal carotid artery, in whom growth of the aneurysm had been observed in two angiograms taken about a week apart the aneurysm had a thin outer wall consisting of part of the pia arachnoid organized by fibroblasts and macrophages (Fig 6)

Neoformation of Small Vessels in the Walls of the Aneurysms

Special attention was paid to the problem whether the aneurysmal wall had an adequate blood supply which might resist degeneration due to lipid infiltration An attempt was made to have the small aneurysmal vessels in 10 aneurysms filled with dye With the dissecting microscope dark streaks were visible suggestive of the presence of adventitious vessels Micro angiography revealed filling of vessels in 4 aneurysms These aneurysms were also studied histopathologically Two of these patients had died of causes other than subarachnoid haemorhage and one patient had never had a bleeding

The vessels differed from the usual *vasa vasorum* in the adventitia of basal vessels in that they extended subintimally In two aneurysms the vessels formed small networks while in the other two only a few vessels were present (Fig 7)



Fig 7 Microradiography of anterior communicating artery aneurysm in 51 year-old patient The aneurysm is cut into two halves Small vessels are present extending from the neck almost to the dome × 15

DISCUSSION

Intracranial aneurysms were studied with reference to the nature and extent of spontaneous repair processes In a few aneurysms, adventitious nerves reached out to the aneurysmal neck containing a semi atrophic media They may have constricted the lumen of the neck decreasing the tendency to bleeding This mechanism would explain why some rare aneurysms do not fill at angiography, however spastic aneurysm carrying vessels do fill The aneurysms may fill 1 or 2 weeks later in control angiography This may depend on release of a spasm of the neck or on solution of a thrombus It is possible that in older patients in whom atheromatous changes are most apt to infiltrate the vessel walls the reaction of spasm may take a somewhat milder course (*Fletcher et al* 1959) The ability for constriction may be less effective and thus the haemorrhages more drastic. On the other hand, atheromatosis may lead to circulatory insufficiency The collateral circulation is less satisfactory in older patients (*Gurdjian et al* 1965) In general spasm is a kind of protective reaction giving a chance for spontaneous organization to strengthen the aneurysmal wall in the early posthaemorrhagic period Spasm affects the aneurysm carrying vessels but apparently it seldom closes the neck of the aneurysm

The following types of repair processes were found Connective tissue membranes outside the aneurysm forming sacs completely or partly investing it or, on the other hand membrane formation inside the aneurysmal wall covering the fundus or one side of the aneurysm Occasionally, the pia arachnoid may be involved in the formation of a new outer wall Such fairly thick pia arachnoid becomes involved when blood components infiltrate the meshwork of the arachnoid Membrane formation inside the aneurysm again starts with blood clotting on the wall Inside the aneurysms blood has been circulating for some time, and blood platelets phagocytes and fibrin are laid down in layers on the inner surface of this coat of clotted blood Later, organization occurs with fibroblasts and collagen Also ruptures in the aneurysmal walls may be healed by fibrosis

Many of the atheromas and some of the calcifications found in the aneurysmal walls were readily separable from the walls Presumably some of the Sudan positive atheromatous material may dissolve, giving turbulent blood flow an opportunity to detach the rest of the atheroma at least partly from the wall after which the wall at this point remains very thin *Crompton* (1966) found that the tendency to recurrent haemorrhages was similar in aneurysms with many outpouchings and those with few outpouchings

In rare instances the aneurysm may be filled with a lipidloaden yellow thrombus which apparently does not dissolve at all Similarly, in rare instances the aneurysm may be completely calcified and thus remain unruptured

It seems that no regeneration of elastin or smooth muscle cells is possible, but the connective tissue wall may be nourished by deep seated neoformed *vasa vasorum* or by pial vessels and if so, it is possible that the wall can withstand the pressure and that rupture due to lipid deposits is prevented

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These studies have been supported by the *Sigrid Juselius foundation*

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BRIEF REPORTS

THE ULTRASTRUCTURE OF TATTOO MARKS

H E Christensen and *H Schmidt*

The patho-physiology of tattooing was first de

show diffusely scattered granules of dye located extracellularly in the the dermis without any in flammatory reaction If allergic reactions occur, e g to mercury in red tattoos or to chromium in green tattoos a dense cellular infiltrate consisting of numerous histiocytes and lymphocytes, scattered foreign body giant cells and varying number of eosinophils and plasma cells will be seen to be mingled with the pigment granules Some of the histiocytes and giant cells contain small and large lumps of pigment

Recently we described the ultrastructural changes in the hereditary disorder, *Incontinentia pigmenti* (*Schmidt et al* 1972) This disease has earlier been termed, with more or less justification, an autochtoneous tattooing The most pronounced morphological characteristic observed was melanine granules placed in a band like formation rather deep in the corium within mesenchymal cells Generally the granules were closely packed in large cytoplasmic clusters which sometimes were mem brane limited It thus seemed quite obvious also to study the location of the dye granules in ar tificial tattooing by electron microscopy The re sults of this investigation are reported in the present paper

Material and methods

The patient who supplied us with a biopsy from

several years ago and never had shown any sort of reactions besides the 'normal' skin irritation just following the procedure of tattooing The colour was blue, i e most probably cobalt aluminate (*Schmidt* 1967), and the location was on the left forearm

One half of the skin biopsy was used for light microscopy Fixation for 24 hours in 10 per cent buffered formaline was followed by conventional histological preparation techniques

The other half was used for electron microscopy after immediate fixation in 3 per cent glutaral dehyde in cacodylate buffer pH 7 2 for 22 hours followed by washing in sucrose in the same buffer The tissue was cut in small pieces, the largest diameter measuring 1 mm, fixed in 1 per cent OsO_4 phosphate buffer for 2 hours and after dehydration embedded in Epon One micron thick sections for orientation purposes, and thin sections for electronmicroscopy were cut in a LKB III ultramicrotome Poststaining was done with zinc uranyl acetate and lead hydroxide, and the sections were examined in a Hitachi HS 8 electron micro scope

The 1 μ thick plastic sections were stained with toluidine blue, and the ordinary histological sec tions with toluidine blue, haematoxylin eosin, van Gieson method, Fontana Masson, alcian blue PAS, methylgreen pyronine (Unna Pappenheim) and Weigert's elastin stain

Results

Light microscopy showed a normal epidermis and stratum papillare In a band like zone situated between the upper one third and the deeper two thirds of the corium rather many vessels, small arteries and veins were present These were often in a perithelial arrangement surrounded by pigment-filled cells or macrophages containing the pigment substance No inflammatory reaction of subacute or acute allergic appearance was found and no granulomas were observed No pathological fibrosis, no abnormalities in elastic membranes and no affected accessory organs were seen

The findings in the 1 μ thick Epon embedded sections confirmed the above mentioned findings

Received 24 ii 72 from *Odense University Hospital University Institute of Pathology and University Department of Dermatology and Venereology Denmark*

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The sections were used for orientation and accordingly, suitable regions were selected for ultrathin sectioning

Electron microscopy revealed vessels with rather thick walls and prominent but normal non pigmented endothelial cells Most of the pigment loaded cells were found near to, but without direct contact with the vessels (Fig 1) These pigment containing cells (tattoo cells) were large elongated cells (Fig 2) The outer cell membrane often showed villous elongations which as slender projections, ran for long distances throughout the tissue These processes could intermingle with those of other cells to such an extent that a resemblance with the foot processes of podocytes of kidney glomeruli was obtained when the processes abutted upon neighbour cells These cell projections were never found to contain pigment substance The pigment loaded macrophages had round, rather small nuclei with a large centrally placed nucleolus and the chromatine was rather sparse with a peripheral condensation at the nuclear membrane The perinuclear space was evident but appeared quite narrow and empty The dye granules from the tattoo material were found in large collections of irregular shape Often these collections were limited by membranes of varying thickness None of the pigment granules could be identified as melanin Occasionally the cytoplasm of the cells contained large vacuoles which (Fig 1) appeared empty or contained a small amount of filamentous material Generally the cytoplasm contained a normal number of mitochondria with normal morphology Sometimes, however large round formations filled with a homogenous rather electron dense substance were also present and some of these lysosome-like bodies showed remnants of mitochondrial cristae The Golgi apparatus was not a prominent feature of the cells studied (Fig 2)

In two fields of view, portions of mast cells were encountered These mast cell fragments contained typical mast cell granules which at higher magnification showed the specific fingerprint like lamellations In the center of these cell portions, quite large accumulations of tattoo granules of the type seen in the macrophages or histocytes were noted (Fig 3)

Discussion

The ultrastructural investigations of a skin biopsy from an old tattoo mark have shown that the foreign material was located in a band like collection of macrophages which tended to be situated perivascular to the slightly increased number of vessels in a zone between the upper and middle third of the corium In the individual cells the pigment accumulations, sometimes membrane bound, occupied the major part of the cytoplasm Some round dense cytoplasmic bodies resembled degenerated mitochondria No pigment was ever observed in any of the numerous projections on the cell surfaces, indicating apparently that these processes do not participate in the transport of pigment from cell to cell Exactly how dye granules are transferred to new generations of macrophages is at present unknown Phagocytosis seems an obvious explanation We are of the opinion that our finding of mast cell cytoplasm with dye pigment granules shows that the mast cells in this tissue have shown active phagocytosis According to *Selye* 1965, however phagocytic activity of mast cells has been a much debated problem Erythrophagocytosis is known to occur in mast cells but

Fig 1 Electron microscopic appearance of several pigmented tattoo cells in apposition to a small vessel (arrows) Two of the cells contain large vacuoles (V) The dark streaks running throughout the tissue between collagen bundles are long slender cell elongations The vertical light lines are knife marks which are difficult to avoid because the accumulations of pigment granules tend to damage the cutting edge × 3700

Fig 2 Tattoo-cell lying in dermal connective tissue The cell has a villous outer cell surface, and the cytoplasm is heavily filled with pigment granules in large accumulations (arrows) A single "myelin figure" (My) is seen At some distance from the cell and parallel to its surface narrow cell projections are seen × 12 400

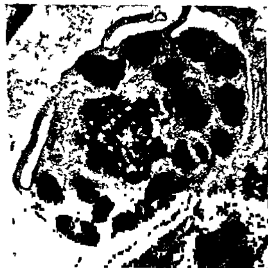


Fig 3 Part of a mast cell (see text) with typical mast cell granules and accumulated pigment material in centre × 27 300

also blood borne soluble metal complexes can be bound by intracellular mast cell granules *Spicer* 1960 reported that the mast cell possibly might have a role in phagocytizing processes He observed siderosis associated with increased lipo fuscins and mast cells in ageing mice, and stated that the intimate association with iron and lipofuscin-laden macrophages deserved attention He found that the distribution of the two cell types was so similar that they often were in contiguity

Finally it shall be mentioned that the tattoo pigment did not show birefringence by polarization microscopy, nor was it auto-fluorescent by fluorescence microscopy

Pigment incontinence (melanin) was not found in any of the preparations However, further studies with the use of microspectrophotometric measurements are in progress in order to identify the different compounds in tattooing In addition, a new case presenting a severe granulomatous reaction which may occur after tattooing (see *Schmidt* 1967), has been studied by electron microscopy The results will be published in a more extensive report (5)

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Acta path microbiol scand Section A 80, 577-588 1972

PATHOGENESIS OF C-CELL NEOPLASIA IN THYROID GLAND

C Cell Proliferation in a Case of Chronic Hypercalcaemia

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Hypertrophy and focal nodular hyperplasia with tumour like proliferation of the C-cells was observed in thyroid tissue removed from a 64 year-old male with non toxic adenomatous goitre and primary hyperparathyroidism The patient was found to have an enlarged para thyroid gland consisting exclusively of waterclear cells It appeared likely that chronic hypercalcaemia had been responsible for the C-cell proliferation Morphological similarities between the thyroid change of the present case and the early stages of some forms of medullary thyroid carcinoma suggest that this neoplasm could arise from hyperplastic foci of C cells It is emphasized that chronic hypercalcaemia may be *one* aetiological factor in human C-cell neoplasia including some types of medullary carcinoma This implies that medullary carcinoma might have been secondarily induced by the parathyroid adenoma in some of those patients in whom both of these manifestations have been encountered

The C cells (*Pearse* 1966) which have long been recognized in mammalian thyroid gland are now generally regarded as normal en docrine cell constituents of this organ, re sponsible for the production of the poly peptide hormone calcitonin (*Bussolati and Pearse* 1967) Most of the present informa tion concerning the histological and biolo gical characteristics of these cells has been derived from animal studies Our knowledge of the normal C cell in man is still rather incomplete because of the difficulty in finding the cells which in turn is mainly due to their small number and uneven distribution within the human thyroid gland A second population of human non follicular cells has been reported, having topographical, mor phological and histochemical properties which are different from those of the C-cells and the true nature of which still remains to be defined (*Ljungberg* 1970 a, 1970 b, 1970 c, 1972 b) Pathology of human C cells has been almost exclusively restricted to medullary thyroid carcinoma. This neoplasm was defined in 1959 by *Hazard et al* as a distinct clinical and pathological entity and has since been found to produce large amounts of calcitonin (*Cunliffe et al* 1968, *Meyer and Abdel Bari* 1968, *Milhaud et al* 1968, *Tashjian and Melvin* 1968) and to contain cells with ultrastructural (*Braunstein et al* 1968, *Meyer and Abdel Bari* 1968) as well as immunohistochemical characteristics (*Bussolati et al* 1969) suggesting that it could arise from C cells

It is well established that calcitonin, and therefore C cells, are involved in calcium homeostasis, though their importance under physiological conditions still remains to be evaluated Studies on various mammalian

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and non-mammalian animals clearly indicate that experimentally induced hypercalcaemia in these animals increases the rate of secretion of calcitonin from the C cells and may induce morphological changes of these cells We recently had the opportunity to study a case of primary hyperparathyroidism with longstanding hypercalcaemia in which there was a marked C cell hyperplasia with a tumour-like proliferation within a very small part of the thyroid parenchyma This report describes this thyroid lesion and the significance of our findings is discussed with respect to the pathogenesis and aetiology of C cell neoplasia

CASE HISTORY

Male, born 1907 In 1908 and 1917 he was treated for an undefined affection of the knee which caused a reduced mobility of the left knee and mild atrophy of the left thigh and calf muscles —In 1968 he was treated surgically for papillary carcinoma of the bladder Postoperatively, he developed a bleeding ulcer of the stomach which was managed conservatively Retrospectively, it was found that the patient during this hospital admission had several serum calcium values above 6 mEq/l with a maximum of 6 8 mEq/l During 1969 and 1970 he had several bouts of gastric distress and in November 1970 there were radiological signs of gastric ulcer

Pains localized to the back and legs started in 1966, and gradually increased In April 1970 he was referred to the department of orthopaedic surgery Radiologically, there were signs of osteopenia in the axial skeleton and a small radiolucent area in the left distal tibia Serum calcium was at this time at the upper limit of normal, 5 4 mEq/l serum inorganic phosphate low 2 1 mg per cent, and alkaline phosphatases borderline, 9 U Biopsy specimens from the tibial lesion and from crista iliaca contained no tumour on histological examination Convincing signs of *osteitis fibrosa* were lacking, but the osteoid seams were increased and osteomalacia was suspected When these tests had been analysed, the patient was seen again in the beginning of June 1970 Another sample of blood for serum calcium was drawn and treatment with calciferol (vitamin D_2) in a dose of 70 000 IU started together with calciumphosphate tablets The serum calcium was now 6 7 mEq/l The calciferol regimen was discontinued after 6 weeks and serum calcium remained between 6 0 and 6 7 mEq/l Treatment with calcium phosphate was however, continued and additional serum calcium values were found to remain in the same range Serum inorganic phosphate decreased to 1 2–1 3 mg per cent In December 1970 he was referred and admitted to the department of endocrinology

The endocrinological evaluation led to a diagnosis of primary hyperparathyroidism on the basis of hypercalcaemia (6 6–6 8 mEq/l), hypophosphataemia (1 9 2 9 mg per cent) and slightly raised levels of alkaline phosphatases (11–13 U) There was no evidence of any other cause of the hypercalcaemia The renal function was slightly impaired with an endogenous creatinine clearance of 71 ml per min and a maximum specific gravity of the urine of 1 014 Radiologically, there were small calcifications in both kidneys A non toxic multinodular goitre was also found At operation in January 1970, an enlarged parathyroid gland was removed from the posterior upper aspect of the left lobe of the thyroid A bilateral subtotal thyroidectomy was also performed Two additional parathyroid glands were identified They were judged as normal and left *in situ* Postoperatively, serum calcium fell to normal levels (4 4–4 7 mEq/l) and serum inorganic phosphate increased to a level of 2 5 mg per cent

No clinical signs of phaeochromocytoma have been noticed in this patient Urinary excretion of mandelic acid (4 2 mg/24 h) as well as free catecholamines (noradrenaline 13 6 µg/24 h adrenaline 2 4 µg/24) was normal There was no family history of parathyroid thyroid or adrenal disease

HISTOCHEMICAL METHODS

The parathyroid and thyroid specimens removed ...nd

of the C cells, the following methods were used the argyrophil silver procedure of *Grimelius (1968)* on sections postfixed in Bouin s fluid as well as the HCl metachromatic staining method introduced by *Solcia et al (1968)*, using Azur A (Chroma AG Stuttgart Unterturkheim Germany) and pseudoisocyanin (N N –diethyl 6 6 –dichloro-pseudoisocyaninchloride, *Dr H Harms*, Leverkusen Germany) on sections postfixed in GPA (glutaraldehyde picric acid acetic acid see *Solcia et al* 1968) and the cresyl violet stain (Kresylviolett E Merck AG, Darmstadt Germany) on sections only fixed in formalin and not subjected to acid hydrolysis (*Ljungberg* 1970 d) Sections stained with pseudoisocyanin were also examined by fluorescence microscopy (Leitz Orthoplan fluorescence microscope with illuminator a *m* Ploem Philips CS 200 lamp 2 mm excitation filter BG 36 + interference filter S 546 with dichroic beam splitting mirror TK 580 + built in suppression



Fig 1 Part of the enlarged parathyroid gland, consisting exclusively of waterclear cells, arranged in alveolar structures.—*Fig 1 a* × 28, *Fig 1 b* × 180 Haematoxylin erythrosin

filter K 580 and suppression filter K 610 in slide Sections on ordinary glass) The alkaline Congo red (*Puchtler et al* 1962) and van Gieson stains were used for the demonstration of amyloid Finally, sections of the thyroid and parathyroid specimens were stained with the method of Masson Hampert (*Romeis* 1948) for demonstrating argentaffin cells

FINDINGS

The parathyroid specimen was an enlarged, encapsulated structure, weighing approximately 1 gram, deep tan in colour Histologically, it consisted entirely of waterclear cells with regular, polyhedric shape and small strongly basophil nuclei The cells were mainly arranged in alveolar structures, separated by thin septa of stroma (Fig 1 a and b) The structure was surrounded by a thin fibrous capsule No normal parathyroid tissue was encountered, thus it could not be classified as adenoma or primary hyperplasia of waterclear cells It contained occasional mast cells, but no cells with C cell characteristics Scattered argentaffin cells, some of which showed long cytoplasmic processes were encountered These cells were situated in the connective tissue stroma or were intermingled with the parenchymatous waterclear cells

The thyroid specimens from both lobes contained several benign follicular adenomas In one block from the left lobe, there was an ill-defined area within the thyroid parenchyma, measuring less than 3 mm diameter where the amount of stroma was increased and which contained a focal accumulation of C-cells These cells were rich in cytoplasm which was often highly vacuolated and split up into rags They were situated singly and in varied sized clusters between the follicles, some however also in *intrafollicular position*,

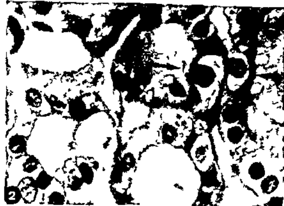


Fig 2 Thyroid follicles with scattered hypertrophied C cells showing ' balooning , pale staining, sometimes vacuolated cytoplasm and large, usually pale staining nuclei Some C cells appear to be separated from the follicular lumen by compressed tree follicular epithelial cells (arrows) × 680 Haematoxylin erythrosin

usually being clearly distinguishable by their abundant, pale staining cytoplasm, from the follicular epithelial cells (Figs 2 and 3) All stages were seen, from small inter- or intra follicular groups of a few cells surrounded by a basement membrane, to large, well circumscribed nodules of numerous C-cells (Figs 3c and d) Sometimes smaller clusters of C-cells surrounding a follicle had coalesced, carrying in the central part an atrophic, degenerating follicle, built up of atrophic true follicular cells on an apparantly intact basement membrane (Fig 3b) The C cells of the smaller clusters usually had abundant pale-staining, vacuolated cytoplasm with rather large, pale nuclei, sometimes excentrically localized and showing a delicate chromatin network with one or several small nucleoli With increasing size of the clusters, an increasing proportion of the cells showed small dense nuclei and a more granulated and eosinophil cytoplasm which was frayed, thus giving the nodule a loose reticular appearance (Fig 3b) The number of C-cells in the walls of the follicles likewise varied from one follicle to another In some, isolated C cells with abundant, clear cytoplasm were seen pushing aside the adjacent follicular cells, but usually a thin rim of follicular epithelial cytoplasm separated the C cell from the lumen of the follicle (Fig 2)

In other follicles, the proliferating C cells had formed crescent like thickenings of the walls or minute nodules, bulging into the follicular lumen (Fig 3c) Occasional follicular structures seemed to be built up entirely of C-cells (Fig 3d) Whether these had been formed *de novo* by C-cells or had been true follicles whose epithelial cells had been destroyed and totally replaced by proliferating C cells could not be determined

Occasional C-cells were encountered in *other parts of the same section*, remote from the lesion described above, but there were no accumulations of the cells in these areas Sections from the other thyroid specimens removed were totally devoid of C cells

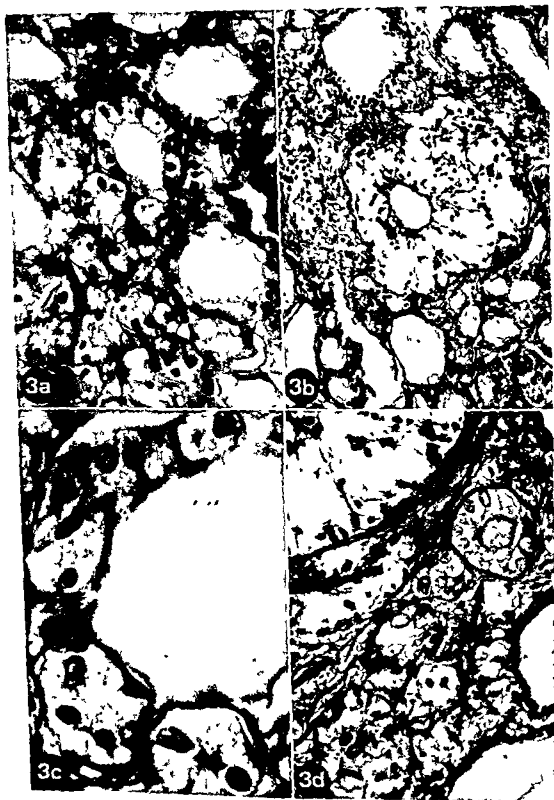
Some of the cells under discussion displayed a distinct metachromasia with HCl Azur A and cresyl violet (Fig 4a), though the reactions were weaker than those generally found in normal human C cells Usually, the smaller granulated cells of the larger clusters reacted most strongly, while the metachromasia was weak or absent in the larger, vacuolated cells lying singly or in small groups The metachromasia with cresyl violet was generally somewhat stronger than that produced by the HCl-Azur A technique, possibly because the fixation had been less suitable for the latter method With HCl pseudoisocyanin, some cells of the larger clusters displayed a red bluish metachromasia in polychromatic light and the same cells emitted a reddish light when examined by fluorescence microscopy under the optical conditions used (Fig 4b) The majority of

Fig 3 Fig 3a Increased number of scattered pale staining cells in intra and interfollicular positions (× 450)

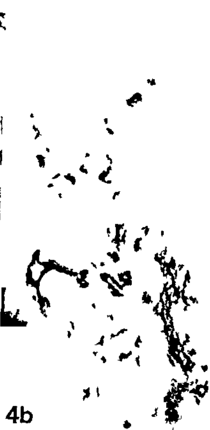
Fig 3b Extrafollicular C cells forming a large loose nodule, carrying in its centre a degenerating follicle (× 190)

Fig 3c Minute nodules of pale staining *C-cells* in the wall of a follicle and bulging into *its lumen* (× *1020*)

Fig 3d A *follicular structure* appears to be entirely built up of C-cells (arrow) Varied sized nodules of C cells are situated in the connective tissue stroma (× 400) Haematoxylin-erythrosin


3a
3b
3c
3d


4a


4b


4c


4d

the C cells reacted unequivocally with Grimelius' argyrophil silver method (Figs 4c and d), though the amount of blackened cytoplasmic material varied from one cell to another

No amyloid was detected within the clusters of C cells

The C cells were non argentaffin On the other hand, there were occasional argentaffin, parafollicular cells which were different from the C cells They were more diffusely distributed than the C cells, also being found in the other sections which were totally devoid of C-cells and they did not form focal accumulations as these cells did They had a characteristic shape, often with long cytoplasmic processes which were usually oriented concentrically to the walls of the follicles They never occurred in intrafollicular position They stained orthochromatically with HCl Azur A, HCl pseudoisocyanin and cresyl violet and they displayed an ambiguous reaction with the Grimelius argyrophil silver technique

DISCUSSION

It appears from the histochemical findings that the thyroid lesion described here represents proliferating C cells The reduction of the metachromatic and argyrophil, granular material in some of the cells as compared with normal human C cells, is consistent with the findings in hypercalcaemic animals (*Kameda* 1970) and in animal C cells cultured in media with a high calcium concentration (*Bussolati et al* 1970) and is believed to be related to discharge of hormon elicited by a high calcium stimulus

Changes of the C cells similar to those found in the human case described here, have been reported by *Williams* (1966) to occur spontaneously in the thyroid gland of old rats He observed different stages of development from hyperplastic foci of C-cells to true tumours As regards the development of the tumours in this animal, *Williams* considered the principal change to be a formation of lobular structures, resulting from C-cell proliferation around follicles, separating the follicular epithelium from the basement membrane and finally destroying the follicles In some parts within the lesion in our human case, the C cells merely showed hypertrophy and hyperplasia At the other end of the spectrum of the proliferation, these cells had formed rounded nodules, entirely built up of C-cells which had arisen from intra- and extrafollicular positions and which sometimes included degenerating follicles These nodules were associated with an increased amount of connective tissue stroma and they had caused a definite destruction of some follicles This undoubtedly suggests a neoplastic change rather than merely hyperplasia, though it appeared impossible to decide the point where the hyperplasia ended and the neoplasia started The lesion bears a strong resemblance to the early stages of some forms of human medullary carcinoma This is particularly evident in the familial form which may serve as a model for study of the mechanism of development of at least this type of the neoplasm (*Ljungberg* 1972 a) In the thyroid glands from 14 cases of familial medullary carcinoma, multiple separated foci of proliferating hyperplastic or neoplastic C cells were found in different parts of one and the same gland and which illustrate the various stages of the development from C cell hyperplasia to true tumours and in which exactly the same morphological patterns of the changes could be recognized as in the minute lesion of the case under discussion (Fig 5a d) In these patients, however, the changes had elsewhere progressed further, *i e* to gross tumours with unequivocally malignant features, such as splitting up the margins of the tumour nodules, infiltration as well as vascular invasion and metastatic growth Fea-

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Fig 4 Fig 4a Metachromatic C-cells stained with cresyl violet (× 440)

Fig 4b Clusters of C-cells displaying pseudoisocyanin fluorescence (× 180)

Fig 4c and *d* Argyrophil C-cells stained according to Grimelius (× 180 and × 450 respectively)

tures of this end stage were not encountered in the case reported here Our present knowledge of the pathogenesis of familial medullary carcinoma clearly suggests that at least some forms of medullary carcinoma may start in the form of uni or multifocal C cell hyperplasia and the possibility must be considered that the thyroid lesion of the present case if untreated could progress into a malignant growth, i e a medullary carcinoma

Argentaffin cells have been reported to occur in increased number in non tumourous thyroid parenchyma and in parathyroid glands in cases of medullary carcinoma and in cases of primary hyperparathyroidism (*Ljungberg* 1972 b) Though these cells were not found to occur in focal accumulations of the kind formed by C cells or within the C cell nodules in the case under discussion their occurence in medullary carcinoma and in metastases of that neoplasm clearly suggests that they may constitute an integral part of the tumour (*Ljungberg* 1970 a 1972 b) Our understanding of their role in the pathogenesis of medullary carcinoma however, must await an elucidation of their biological nature and relationship to the C cells

Studies on various mammalian and non mammalian animals indicate that alterations of the calcium homeostasis are associated with cytological changes in the C cells Thus experimentally induced hypercalcaemia is reported to be associated with a rapid and significant decrease of the number of their specific cytoplasmic granules (*Matsu aua* 1966 *Pearse* 1966, *Ericson* 1968, *Lietz* 1970) These granules are believed to be the site of the storage form of calcitonin (*Bauer and Teitelbaum* 1966, *Kalina and Pearse* 1971) Hypercalcaemia of sufficiently long duration has been reported also to induce hypertrophy and hyperplasia of the C cells in several mammalian species (*Walker* 1966 *Rohr and Hasler* 1968, *Capen and Young* 1969, *Kameda* 1970), in avian species (*Cipera et al* 1970) and in amphibian (*Robertson* 1968) species *Capen and Young* (1969) studying the time course of the effects of hypercalcaemia induced by administration of vitamin D in cows noticed a marked hypertrophy of the C cells in cows receiving the vitamin for 5 days while hyperplasia was not evident until the 30th day of hypercalcaemic stimulation The morphological changes were associated with a significant reduction in the calcitonin content of the thyroid glands (*Young and Capen* 1970) Recently *Krook et al* (1969) and *Wilkie and Krook* (1970) reported that C cell tumours were frequent thyroid tomours in bulls while they were totally absent in cows They suggested that high level of dietary calcium might be the most important aetiological factor in the development of this tumour in bulls eliciting a state of 'hypercalcitonism which might also explain the high incidence of osteopetrosis in these animals The absence of thyroid C cell tumours as well as the low incidence of osteopetrosis in cows on the other hand was thought to reflect the relative lack of stimulus for production of calcitonin in animals using dietary calcium for foetal growth and lactation

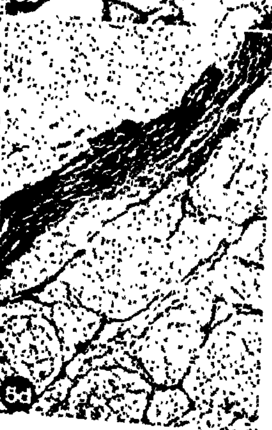
In the light of this information it seems reasonable to assume that morphological changes in the C cells could also occur in man under conditions associated with hypercalcaemia *Kracht et al* (1970) state that C cell hyperplasia may occur in various forms of hyperparathyroidism in man Thus it seems probable that the focal change of the C cells in the human case reported in this paper is the result of a chronic stimulation of these cells brought about by a hypercalcaemia known to have been present for at least two years Since medullary carcinoma can sometimes arise from hyperplastic foci of C cells there is a distinct possibility that

Fig 5 Thyroid gland with medullary carcinoma (fam l al form) Remote from the gross tumours are multiple varied sized and circumscribed clusters of proliferating C-cells (*Figs 5 a b* and *c*) illustrating various stages of development *to the* gross unequivocally malignant tumour (*Fig 5d*) —*Fig 5a* × 448 *Fig 5b* × 70 *Fig 5c* × 180 and *Fig 5d* × 28
van Gieson.


6a


6b


6c


6d

chronic hypercalcaemia is *one* aetiological factor in human C cell neoplasia, including some forms of medullary carcinoma

Chief cell hyperplasia or adenoma of the parathyroids have been reported to occur in association with medullary thyroid carcinoma The current view on the patho-physiological relation between these two lesions is that of a secondary hyperparathyroidism developing to counteract the hypocalcaemic effect of excessive calcitonin production by the thyroid neoplasm (*Schimke et al* 1968, *Steiner et al* 1968, *Bartlett et al* 1971, *Catalona et al* 1971) Since an inverse feedback relationship exists between the serum calcium level and parathyroid hormone production, this may result in normocalcaemia, which is the most frequent finding in patients with medullary thyroid carcinoma Overstimulated parathyroid glands may however, become autonomous producing inappropriately large amounts of parathyroid hormone This phenomenon may account for the fact that some patients with medullary carcinoma have had hypercalcaemia, which was normalized by parathyroid resection (*Manning et al* 1963, *Steiner et al* 1968)

The experimental data mentioned above clearly suggest that the reverse order of the patho psysiological events is possible, *i e* a C-cell proliferation secondarily induced by a hypercalcaemic state This is the most likely possibility in our patient, where clinical evidence strongly suggested that the basic disorder was primary hyperparathyroidism with chronic hypercalcaemia In this regard it is noteworthy that *Melvin et al* (1971) studying the serum calcitonin and parathyroid hormone levels in members of a family afflicted with medullary carcinoma and phaeochromocytoma, found that several of the young members had shown intermittently elevated serum parathyroid hormone levels despite normal concentrations of circulating calcitonin

Supported by a grant from the *Board for Medical Research of Swedish Life Insurance Companies*

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ARGENTAFFIN CELLS IN HUMAN THYROID AND PARATHYROID AND THEIR RELATIONSHIP TO C-CELLS AND MEDULLARY CARCINOMA

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Patients with medullary thyroid carcinoma may show two histochemically different populations of non follicular cells in those parts of the thyroid parenchyma as are not involved by the neoplasm Besides cells with characteristics consistent with C cells (basic dye metachromasia and argyrophilia), there is a second topographically and morphologically distinct population of extra follicular cells which show argentaffin and orthochromatic cytoplasmic granules These cells were found in 13 out of 25 cases of medullary carcinoma and they were also present in parathyroid glands in 5 out of 8 of these patients Their malignant counterparts in the tumours were found in 20 out of 34 cases Argentaffin and orthochromatic cells were only sporadically seen in a series consisting of various benign thyroid diseases and they were totally absent in cases of malignant neoplasms other than medullary carcinoma The nature of the argentaffin (orthochromatic) cell is enigmatic. It may be related to the C cell but its characteristic morphology and topography suggest that it is a distinct type of cell with different functions

Since the definition of medullary carcinoma of the thyroid gland by *Hazard, Hawk and Crile* in 1959, this tumour has been widely accepted as a distinct clinical and pathological entity During the past few years considerable evidence is accumulating to suggest that the neoplasm also has a distinct histogenesis On the basis of comparative morphological studies, *Williams* (1966) proposed that medullary thyroid carcinoma was derived from parafollicular cells, which are now considered as the calcitonin producing cells of the thyroid gland and commonly referred to as 'C-cells (for review, see *Lietz* 1971) Later it was realized that cells in the tumour show ultrastructural similarity to the mammalian calcitonin secreting cells (*Braunstein et al* 1968 *Meyer & Abdel Bari* 1968) A number of recent accounts where calcitonin has formed a principle product of the tumour as well as the immunofluorescent localization of this substance in a large number of the tumour cells (*Bussolati et al* 1969) provide further support to this view

Familial medullary carcinoma has been found to contain a small population of cells which differs from the main mass of cells in being argentaffin and in having a characteristic spider like shape (*Ljungberg* 1970 a) Argentaffin cells in parafollicular position have also been demonstrated in the non-tumorous parenchyma of a thyroid gland with medullary carcinoma (*Ljungberg* 1970b) These cells showed close morphological similarity to the argentaffin cells in the tumours They differed from the C cells with respect to morphological and certain topographical

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and histochemical characteristics (*Ljungberg* 1970 c) The findings in this case prompted further studies on the occurrence of these cells in additional cases of medullary carcinoma with special emphasis on their relationship to the C-cells as well as their occurrence in glands involved by other types of thyroid carcinoma, various non neoplastic thyroid diseases and in normal glands

MATERIALS AND METHODS

Specimens from 34 thyroid glands with medullary carcinoma were used Fourteen of these were of the familial type Specimens from thyroid glands affected by other thyroid diseases as well as from normal glands were also studied These included 36 surgical and 4 autopsy cases of malignant thyroid neoplasms other than medullary carcinoma (*viz* *papillary, follicular and anaplastic carcinoma*) and surgical material consisting of 30 cases of benign thyroid lesions (*viz* follicular [illegible]

[illegible] *or skeletal diseases, death having been caused* by unrelated conditions The reference material represented age groups ranging from premature *foetuses to old individuals Parathyroid glands from* 8 cases with medullary carcinoma of the thyroid as well as from 16 surgical patients without medullary *carcinoma or parathyroid disease were also examined*

Tissue Preparations

The surgical material had been placed in the fixing fluid within few hours after the operation The specimens taken from autopsy cases were collected from 3 to 24 hours after death The specimens were invariably fixed in 10 per cent buf*fered formalin (pH 7 5–8 0) for 24 to 48 hours* dehydrated embedded in paraffin and sectioned at about 6 μ Certain staining methods required special postfixation (*see below*)

Staining Techniques

Haematoxylin-erythrosin and van Gieson were used for routine histology

Identification of argentaffin cells The method of Masson Hamperl (*Romeis* 1948 a)

Identification of C cells The metachromatic staining technique (*Solcia et al* 1968), using Azur A and pseudoisocyanin (N N'-diethyl-6 6'-dichloropseudoisocyanin chloride, Leverkusen, Germany), was applied on sections which had been postfixed in GPA (glutaraldehyde picric acid acetic acid, *see Solcia et al* 1968) at 60° C for 1 2 hours and hydrolysed in 0 2 N HCl at 60° C for 1–2 hours Cresyl violet was also applied on sections only fixed in formalin and not subjected to acid hydrolysis (*Ljungberg* 1970 d) Pseudoisocyanin fluorescence was examined with a Leitz Orthoplan fluorescence microscope, equipped with a Philips CS 200 mercury burner and exchangeable dichroic beam splitting mirrors a m *Ploem* The activating light was filtered through a 2 mm BG 36 filter combined with an interference, line filter, isolating the 546 nm region The dichroic mirror transmitting above 580 nm was combined with a barrier filter transmitting above 580 nm This arrangement provides good conditions for the visual observation of pseudoisocyanin stained C cells which show a strong yellow fluorescence, confined to cytoplasmic granules, while the background emits a very weak red brown light

Grimelius silver nitrate procedure (*Grimelius* 1968) was used for the demonstration of argyrophilia of C cells (*De Grandi* 1970) Sections from blocks which were found to contain C cells and/or argentaffin cells were further analysed with the following methods

Fluorescence microscopy for autofluorescence in formalin fixed sections which had been deparaffinized and mounted in xylene on slides of ordinary glass The fluorescence microscope described above was used, with exchangeable filter combinations allowing excitation at variable wave lengths

Diazo coupling (*Pearse* 1960 a) and ninhydrin methods (*Barka & Anderson 1963*) for 5 hydroxytryptamine

PAS with and without diastase digestion (*Lillie* 1965 a), long Ziehl Neelsen (*Pearse* 1960 b) and Sudan Black (*Pearse* 1960 c) for lipopigments in dehydrated sections

Prussian Blue and Turnbull Blue (*Romeis* 1948 b) for ironpigments

RESULTS

Medullary Carcinoma

Argentaffin cells Twenty tumours, including 10 of the familial form contained scattered cells whose cytoplasm showed an argentaffin, granular material (*Fig 1 a*) Their shape, relative number and distribution conformed with the argentaffin spider-cells' previously described in cases of familial medullary carcinoma (*Ljungberg* 1970 a) *Their* distribution within the tumour was *patchy* usually being found only in a single or a few *specimens of the tumours most specimens*

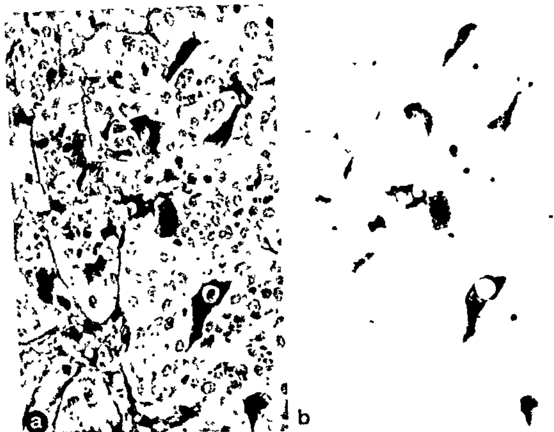


Fig 1 *a* Argentaffin cells in medullary thyroid carcinoma Masson Hamperl *b* The same cells showing autofluorescence in formalin fixed, unstained section mounted in xylene (× 450)

being totally devoid of such cells The majority of the cells in the tumours, however, did not contain any material capable of reducing silver salt spontaneously The argentaffin cells were readily identified in consecutive, haematoxylin-erythrosin stained sections In these, the argentaffin cells showed small hyperchromatic nuclei, often in excentric position and their cytoplasm was filled with closely packed, rather coarse, red brown granules which usually completely hid the nuclei In unstained sections, these granules displayed a weak intrinsic yellowish colour

Argentaffin, extra-follicular cells in non-tumorous thyroid tissue were found in 13 out of 25 glands involved by medullary carcinoma, a total of 7 examples of the familial and 6 of the sporadic form In those parts as were not destroyed by the tumour, there were diffusely distributed cells, usually occurring singly and having a polyhedric, often spider-like shape Their cytoplasm was closely packed with argentaffin granules of approximately the same size as in the argentaffin cells within the medullary tumours The cell bodies were large, some were up to ten times the size of an erythrocyte (Fig 2 a) Most cells, however, were only represented by their slender, cytoplasmic processes, their perikaryons being outside the plane of the section The processes of the argentaffin cells were close and concentric to the walls of the follicles, but apparently outside the follicular basement membrane (Fig 2 b) The cell bodies were lying in the interfollicular stroma, either close to the follicular walls or

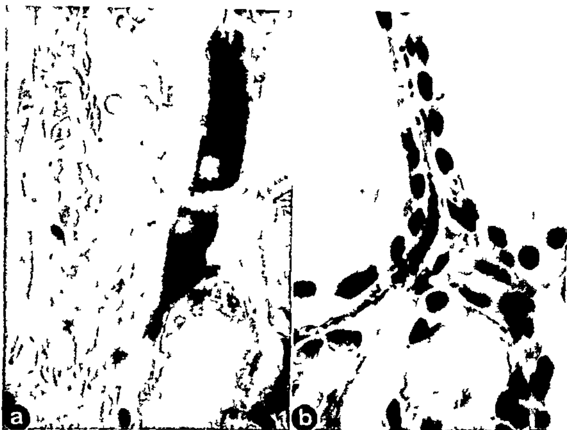


Fig 2 *a* Large cell bod es of argentaffin cells in non tumorous thyroid tissue in a case of medullary carcinoma One cell in close proximity to the wall of a follicle The entire cytoplasm is f lled with closely packed coarse blackened granules *b* Cytoplasm c process of argentaffin cell close to foll cular wall in a part not involved by the neoplasm Masson Hamperl (× 1120)

at some distance from them No argentaffin cells were seen intermingled with the fol licular epithelial cells Some also showed a close topographical relation to the para follicular blood capillaries but they were never seen to come into direct contact with their lumina In haematoxylin erythrosin, the nuclei of the argentaffin cells usually showed a coarse chromatin net work Their cyto plasmic granules were red brown

C cells Some non argentaffin cells in the medullary tumours exhibited a metachroma tic staining confined to a granular cytoplas mic material Similar results were achieved with all three metachromatic staining me thods With Azur A and pseudoisocyanin this required refixation of the sections in GPA and subsequent hydrolysis in strong acid whereas cresyl violet also produced a meta chromatic staining in sections only fixed in formalin and not subjected to acid hydrolysis With pseudoisocyanin the reacting cells showed a red bluish metachromasia when examined in polychromatic light and the same cells fluoresced with a strong yellow colour on a weak red brown background when studied by fluorescence microscopy un der the optical conditions used The majority of tumour cells however contained only little or no metachromatic material regardless of type of dye used The argentaffin cells in the tumours did not show a metachromatic reaction but their cytoplasmatic granules dis played a strong orthochromasia (Azur A dark blue Cresyl violet dark blue violet pseudoisocyanin brick red in polychromatic





Fig 3 Clusters of proliferating C-cells in a part of a gland with familial medullary carcinoma, but remote from the gross tumours *a* van Gieson (× 180) *b* Pseudoisocyanin fluorescence (× 840)

light) The granules were strongly stained whether or not hydrolysis had been performed The argentaffin cells did not fluoresce with pseudoisocyanin A variable number of non argentaffin cells in the tumours displayed argyrophilia with Grimelius' method The argentaffin cells, however, did not react unequivocally, being stained more or less dark brownish with this technique

Varied sized clusters of metachromatic and argyrophil cells were observed in the thyroid parenchyma, remote from the gross tumours in 10 of the fourteen familial cases of medullary carcinoma These cells were located in the interfollicular stroma or in the walls of the follicles, some of which were thickened crescent like Occasional clusters carried in their central parts degenerating follicles In some parts, various foci of such cells were observed, illustrating different stages in the *in situ* development of medullary carcinoma Some cells lying singly or in small groups were hypertrophied with vacuolated cytoplasm Many cells in the larger clusters showed disrupted and frayed cytoplasm which was heavily granulated and reacted strongly with the argyrophil and metachromatic staining methods (Fig 3 a and b) In general, reaction of cells of the microscopical nodules was stronger with these methods than that of cells in the gross tumours The argentaffin cells in the thyroid parenchyma failed to stain metachromatically On the contrary, the granules of these cells were strongly orthochromatic (Fig 4 a) They stained dark brownish with Grimelius' method and they did not fluoresce with pseudoisocyanin They showed no specific orientation in relation to the C cell clusters, being found also in parts which lacked the latter cells Neither did they

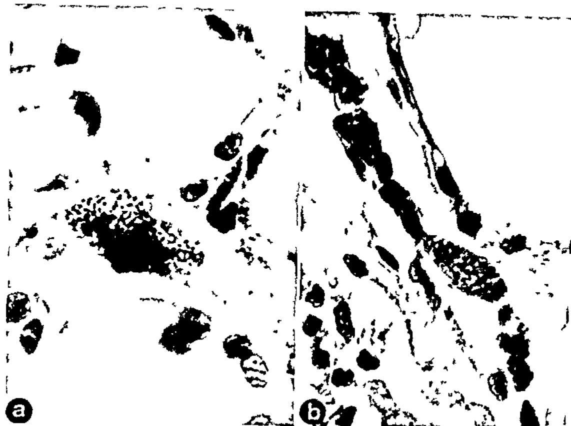


Fig 4 Non tumorous thyroid tissue Same case as in Fig 2 *a* Parts of argentaffin cells stained orthochromatically *b* C-cells in another part of the same section, stained metachromatically Cresyl violet without acid hydrolysis (× 1120)

form focal accumulations as did the C-cells and they were never encountered within the microscopical clusters of C cells C-cells, as defined by their metachromatic and argyrophil staining properties, were only found in non tumorous parenchyma of one of the non familial cases of medullary carcinoma The findings in this case have been described previously (*Ljungberg* 1970 b, c, and d) The C cells in this case occurred within a small area in a single specimen from the lobe which was unaffected by cancer The majority of C cells appeared in interfollicular position, in groups of up to 10 cells but not in larger nodules of the kind seen in the familial cases (Fig 4 b and Fig 5 a, b, c) The argentaffin cells were more diffusely distributed than the C-cells and they were also found in other specimens from the gland Though occasional C cells were situated close to the argentaffin cells, the two populations of cells usually appeared separately at some distance from each other

Other Types of Thyroid Carcinoma Benign Thyroid Lesions and Normal Glands

Argentaffin cells of the kind described above were not encountered in any of the forty examples of other types of carcinoma not related to medullary carcinoma Neither were there any argentaffin cells in those parts of the glands as were not involved by the tumours (27 cases) Occasional cells in the colloid of follicles and in parts with fibrosis and degeneration contained brown to black material in the form of varied sized granules These cells however were readily recognized as macrophages with material



Fig 5 Metachromatic C-cells in non tumorous thyroid tissue from the same case in Fig 2 *a* Cresyl violet *b* Pseudo isocyanin fluorescence *c* HCl Azur A (× 450)

capable of reducing silver salt In some glands the follicular epithelial cells contained granules which were much larger and fewer than those in the argentaffin parafollicular cells and which stained dark brownish rather than black

A few argentaffin cells in parafollicular position were found sporadically in normal thyroid parenchyma in 6 out of 50 cases consisting of normal glands and benign thyroid diseases Three autopsy glands were from children aged ½ to 3 years and the remaining three were surgical cases, all women 35 to 50 years old with benign follicular adenomas Metachromatic and argyrophil cells conforming with C cells were found in 3 autopsy cases a 1 year-old boy and 2 adults 55 and 60 years old The C cells were found in a small part of a single section while other specimens were totally devoid of such cells They were invariably much fewer than in thyroid tissue of glands with medullary carcinoma

Parathyroid Glands

Parathyroid glands available for study in 8 cases of medullary carcinoma were all normal histologically In 5 of these cases argentaffin cells with long cytoplasmic processes were found in the connective tissue stroma, occasional were also intermingled with the parenchymatous cells Some cells were found in the adventitia of blood vessels, they were occasionally situated between the adventitia and the smooth muscle layer of arterioles within the glands (Fig 6) They did not occur in the soft tissue surrounding the glands The cytoplasmic granules of these cells were of almost the same size as those of the thyroidal argentaffin cells and they



Fig 6 Argentaffin cells in the wall of a blood vessel in parathyroid gland from a case of medullary carcinoma Masson Hamperl (× 450)

(3 mm BG 3 filter combined with interference line filter S 405 and dichroic beam splitting mirror TK 455 with built in suppression filter K 460) Under these optical conditions the granules emitted a dull yellow green light on a blue greenish background A fairly long exposure (20 min) of the sections to this light did not bring about any visible decrease in the intensity of the emitted light though the background fluorescence was clearly weakened The non argentaffin cells in the tumours and the C cells in the thyroid tissue were not fluorescening Reactions for 5 hydroxytryptamine were invariably negative in both argentaffin cells and C cells The results of the lipid methods were equivocal Sudan Black did not stain the argentaffin cells even after 24 hours staining nor did the C cells take up the dye The staining with PAS regardless of diastase digestion and with long Ziehl Neelsen were possibly weakly positive in some argentaffin cells in the tumours and in the thyroid parenchyma and parathyroids Iron was not detected in the *argentaffin cells or in the C cells*

showed the same reaction pattern with the other staining methods used in this study Cells with features of C cells were not encountered The glands contained scattered mast cells which could be distinguished from C cells by examining their metachromatic staining at different pH values (*Solcia et al* 1968 *Ljungberg* 1970 c) Argentaffin cells were totally absent in a series consisting of normal parathyroids removed from 16 patients without medullary carcinoma or signs of parathyroid diseases

Other Histochemical Studies

The argentaffin cells in the medullary tumours and in non tumorous thyroid tissue displayed a rather weak autofluorescence confined to their cytoplasmic granules (Fig 1 b) This fluorescence appeared to be strongest when excitation with violet light was used

DISCUSSION

The results reported in this study are consistent with the previously reported view on the occurence of two different populations of cells in cases of familial medullary carcinoma (*Ljungberg* 1970 a) and indicate that these cells likewise may occur in the sporadic form of this neoplasm They also suggest the existence of two morphologically topographically and histochemically distinct populations of human non follicular cells both of which seem to participate in the development of medullary carcinoma *Sandritter and Klein* (1954) described two types of argyrophil cells in the thyroid gland of dog rabbit and man One of these designated as parafollicular cell was most often situated in parafollicular position showed a rounded shape and reacted readily with the argyrophil staining method This may well correspond to the *C cell* The other called interstitial cell was usually

situated at some distance from the follicles showed a spider like shape reminding the authors of a ganglion cell with slender cytoplasmic processes which were often seen closely related to the follicular walls This cell contained granules which were coarser than those of the parafollicular cell and it did not react as readily with the argyrophil silver method as did the former cell The description of the interstitial cell of Sandritter and Klein fits fairly well with the argentaffin cell described here as regards shape size of granules topographical relation to follicular walls as well as its delayed reactivity with the argyrophil silver stain The authors however did not investigate the argentaffinity of their cells and they admit that their findings were not unequivocal as regards the distinction between the two types of cells in the human cases studied

The importance of the argentaffin cells in the development of medullary carcinoma is difficult to determine These cells have invariably constituted a very small part of the total tumour mass the C cell variety building up the major part In glands involved by familial medullary carcinoma multiple foci of C cells have been encountered which suggest different stages of development to true tumours (*Ljungberg* & *Dymling* 1972 *Ljungberg* 1972) Argentaffin cells have never been observed to form focal accumulations of the kind formed by the C cells in these glands nor have they been encountered within such microscopical foci of proliferating C cells Quantitatively their contribution to the formation of the tumours would therefore appear insignificant. On the other hand, argentaffin cells were found in extrathyroidal metastatic tumour This suggests that these cells constitute an integral part of the tumour and do not merel[illegible]

[illegible] g tumour

The nature of the *argentaffin* orthochromatic cell is enigmatic It cannot be ruled out that the non argentaffin metachromatic and argyrophil C cell and the argentaffin orthochromatic cell belong to the same type of cell the histochemical differences then reflecting different functional states in these cells Monoamines occur naturally in C cells of some animals (*Falck et al* 1964) In other including man normal C cells do not naturally contain such substances but the cells are able to take up and to decarboxylate administered monoamine precursors as well as to store the amine thus formed (*Owman* & *Sundler* 1968) Furthermore a small population of cells in cases of human medullary carcinoma has shown specific formaldehyde induced fluorescence of the type produced by certain monoamines (*Falck et al* 1968) A certain proportion of the C cells in the human cases studied here may thus be thought to have stored such substances in a form which will resist considerable extraction during fixation dehydration and staining procedures and cause these cells to be argentaffin Since the argentaffin cells did not react with the diazo coupling or ninhydrin methods substances other than 5 hydroxytryptamine will have to be considered Certain monoamine precursors bound as NH-terminal residues to polypeptides may react with formaldehyde in aqueous solution or in gaseous form under the conditions of Falck Hillarp s method forming fluorescent condensates (*Hakanson* & *Sundler* 1971) The possibility that firmly bound compounds of this kind could be responsible for the argentaffinity as well as for the fluorescence found in the argentaffin cells of unstained formalin fixed sections may also be considered Lipid containing material such as lipofuscins may show argentaffinity with the method used in this study (*Lillie* 1965 b) Carotid body tumours which are biologically related to medullary carcinoma are known to contain many lipofuscin laden cells giving an argentaffin reaction (*Grimley* & *Glenner* 1967 *Capella* & *Solcia* 1971) Such cells also display autofluorescence upon excitation with UV light (*Thompson* 1966 *Capella* & *Solcia* 1971) They may show cytoplasmic basophilia and stain orthochromatically with cationic aniline dyes (*Capella* & *Solcia* 1971) Since

the lipid methods gave equivocal results on the argentaffin cells studied here, it cannot be excluded that the reaction pattern may have been due to the presence of such substances

On the other hand, the characteristic morphology and topography of the two populations of cells suggest that they are really two different types of cells with distinct functions which may well be closely interrelated It is noteworthy that argentaffin cells similar to those in the thyroid, also occurred in parathyroid glands of some of the cases with medullary carcinoma Recent results indicate that, besides in cases with medullary carcinoma, the number of argentaffin cells appears to be increased also in thyroid and parathyroid glands in cases of primary hyperparathyroidism (*Ljungberg & Dymling* 1972, *Ljungberg & Nowak* unpublished observations) This suggests that the argentaffin cell, as the C cell, may be involved in calcium homeostasis

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This study was partially presented at Endocrinology 1971, the Third International Symposium London July 19–22, 1971

Supported by a grant from the *Board for Medical Research of Swedish Life Insurance Company*

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FOA-KURLOFF CELLS IN THE HASSAL BODIES OF OESTROGENIZED GUINEA-PIGS

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Oestrogen treated guinea pigs revealed swollen Hassal bodies containing a few intact Foa Kurloff cells Much amorphous intact material in the Hassal bodies gave staining reactions identical to those found with Foa Kurloff cells including a strong acid phosphatase reaction

Untreated guinea pigs contain a small percentage of Foa Kurloff cells in the peripheral blood and lymphoid tissue (*Marshall* 1956) The cells, which are primarily characterized by a high cytoplasmic content of periodic acid Schiff (PAS) positive mucoprotein (*Ruth* 1958), are selectively produced by treatment with oestrogen (*Ledingham* 1940)

During studies of oestrogen induced amyloidosis (*Ebbesen* 1968) and oestrogen induced thymomas (*Ebbesen & Doenhof* 1971) in mice it became apparent that oestrogenized mice develop cyst like structures which seem analogous to Hassal bodies in other species These cysts contain PAS positive material and cell remnants but electron microscopic examinations have failed to reveal the source of these cells (*Ebbesen & Nielsen* 1972)

The purpose of the present work was to use the characteristic morphology and staining characteristics of the Foa Kurloff cells with a view to determining whether such cells may be present in Hassal bodies of oestrogenized guinea pigs (*Ranlov et al* 1970)

MATERIAL AND METHODS

Ten adult female guinea pigs fed on a diet sufficient in vitamin C were given 0 25 ml *Fol cycl n Ciba* (Oestradiolum NFN) (1 mg/ml) subcutaneously once a week for 6 weeks Ten untreated animals served as controls The animals were autopsied a few days after the last injection and sections stained with haematoxylin-eosine PAS Unna Pappenheim Van Gieson Hansen alkaline Congo red alcian blue pH 2 5 toluidine blue pH 2 0 and 5 0 Burstone s acid phosphatase stain (*Burstone* 1958 *Burstone* 1962) and Lendrum s phloxine tartrazine stain (*Lendrum* 1939)

RESULTS

Oestrogenized guinea pigs exhibited thymuses crowded with Foa Kurloff cells staining with PAS and eosine and in the peripheral cytoplasmic rim with Unna Pappenheim too but not with Van Gieson Hansen and alkaline Congo red

Hassal bodies of oestrogen treated animals appeared larger than those of untreated animals (Figs 1 and 2) The Hassal bodies

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This investigation was supported by *Daell Fonden* and *Anders Hasselbalcks Fond til Leukæmiens Bekæmpelse*

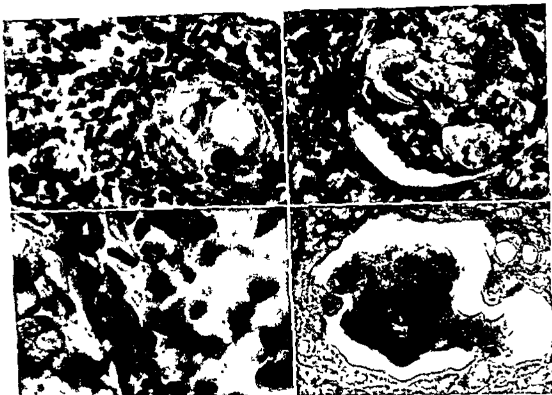


Fig 1 Hassal body of untreated adult guinea pig PAS technique × 840

Fig 2 Slightly swollen Hassal body of oestrogenized adult guinea pig Many Foa Kurloff bodies in the tissue surrounding the Hassal body and a few in the body PAS technique × 840

Fig 3 Larger magnification on the Hassal body depicted in Fig 2 Foa Kurloff bodies are clearly seen both inside the Hassal body (left) and outside (right) PAS technique × 2100

Fig 4 Acid phosphatase reaction showing strong positivity in the Hassal body Burstone technique × 500

furthermore contained a few Foa-Kurloff cells (Fig 3) recognizable by their morphology and staining properties

The amorphous material in the lumen of the dilated Hassal bodies showed exactly the sam[illegible]

[illegible] ortho-chromatic with toluidine blue at the used pH 2 0 and 5 0, positive Lendrum-reaction and strong positivity in the acid phosphatase reaction (Fig 4)

DISCUSSION

Our finding of Foa-Kurloff cells in the Hassal bodies shows that a cell of the lymphoid system (*Marshall* 1956) probably derived from outside the thymus (*Ranlov et al* 1970) may lodge in the Hassal bodies as may other particulate material (*Blau & Veall* 1967) From ultrastructural studies (*Christensen et al* 1971) some immunologic function of the Foa-Kurloff cell must be interfered The present study points to Foa-Kurloff cells as a possible source of a thymus humoral factor (*Metcalf* 1956, *Law et al* 1964) released from the Hassal bodies

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THE PROLIFERATION KINETICS OF L 1210 ASCITES TUMOUR

PER DOMBERNOWSKY

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The proliferation kinetics of leukemia L 1210 ascites tumour grown in DBA/2 × NMRI mice was determined Using the host life span method we found a tumour doubling time of 11 7 hours The doubling time determined by cell counting was 10 hours from day 4–6 25 hours between day 6 and 7 and about 75 hours from day 7–9 with a standard inoculum of 10^5 cells Autoradiography of tumour cells after Thymidine ^{3}H labelling on day 6 after transplantation showed a cell cycle time of 22 hours derived from the per cent labelled mitoses curve The time spent in DNA synthesis was 15 hours Continuous labelling showed a growth fraction very near 1 The discrepancy between our data and the data published by other authors is discussed Neither host life-span of the mice nor cell doubling time were measurable affected with a dose of 1 0 μCi Thymidine ^{3}H per gram of mouse used in the studies

The experimental leukemia L 1210 originated as a lymphocytic leukemia in a DBA/2 male mouse after skin painting with methyl cholanthrene (*Law et al* 1949) This tumour has been used extensively in cancer research during the last few years and has been of great importance in the screening program of The National Cancer Institute in U S A, since the majority of the clinically useful anti tumour agents have been selected by using this tumour (*Leiter & Schneiderman* 1959)

Skipper and his group (1964) have increased our knowledge of the cell kinetics of L 1210 leukemia considerably by establishing a precise quantitative relation between the route of injection, the number of leukemic cells injected and the mean survival time of the animals The doubling time of L 1210 in the ascites form has been determined by cell counting (*Skipper et al* 1964, *Johnson et al* 1965)

A detailed knowledge of the cell cycle and its phases in this tumour is important in view of the marked differences in response, that are found with variation in scheduling of cycle dependent cancer chemotherapeutic agents (*Skipper et al* 1964, 1967)

Until now only sparse information has been published on the effects of cytostatics on the cell cycle of this tumour (*Shirakawa & Frei* 1970, *Young & De Vita* 1970)

It is the purpose of this paper to describe growth characteristics and cell cycle of our strain of leukemia L 1210 ascites tumour in a late stage of intraperitoneal growth in F_1 hybrid mice (DBA/2 × NMRI), and to compare our data with data obtained by other authors using the same technic (*Yankee et al* 1967, *Wheeler et al* 1967, *Skipper* 1968) The study combines growth experiments, the wave of labelled mitoses by pulse labelling per cent labelling of interphase tu

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mour cells by continuous labelling and mitotic index The results will form a basis for a subsequent study on the influence of cytostatic agents on cell cycle parameters

MATERIALS AND METHODS

First generation hybrids of female random bred Swiss mice and male inbred *DBA/2* mice were used in all experiments All mice used were males weighing 18–22 gm They were housed 10 to a cage and given a sterilized dry food, enriched with vitamins and water ad libitum A strain of leukemia L 1210 obtained from Southern Research Institute, Birmingham, Alabama, was used The tumour was maintained in DBA/2 mice in our laboratory since May 1969 by weekly intraperitoneal inoculation of 10^5 cells in a volume of 0 1 ml

The procedure of tumour transplantation described by *Klein & Révesz* (1953) was used with some modifications Ascites tumour cells from one or more animals inoculated 6–7 days earlier were removed under sterile conditions pooled and kept below 4° C The number of cells were counted in a hemocytometer In the suspension used more than 95 per cent of the nucleated cells were tumour cells A viability test with nigrosin (*Kaltenbach et al* 1958) showed in all cases less than 5 per cent dead cells

For the tumour growth experiments, the mice were sacrificed from 4 to 9 days after inoculation The abdomen was opened and the peritoneal cavity washed with isotonic NaCl containing 150 I U Heparin per 5 ml isotonic NaCl Ascitic fluid and wash fluid were pooled, weighed and after dilution the number of cells were counted in a hemocytometer

The mean survival time following inoculation was studied by examining the animals at the same time each day The length of survival of each animal after tumour inoculation was estimated as the time interval between inoculation and the mean of the last time it was seen alive and the day it was found dead

In the high resolution autoradiography Thymidine (methyl 3H) (^{3}H Tdr) with a specific activity of 5 0 Ci/mM (The Radiochemical Centre, Amersham England) was used The amount injected and the time of injection are described in detail in the appropriate sections of the results The injections were given intraperitoneally after

volume of 0 2

citic fluid was

ity Cells were

smeared on cleaned gelatin-coated glass slides, air dried and then fixed in Carnoys fixative for five minutes Smears were dipped in Ilford K2 nuclear emulsion diluted 1 2 with water stored at 4° C for 2 weeks and then developed in D 170 amidol developer for 5 minutes at 18° C

Mitotic indices were determined by counting 3000 labelled or unlabelled tumour cells and the number of labelled mitoses on 100 mitotic cells Only metaphases and anaphases were included The per cent of labelled cells was determined on 1000 tumour cells In all experiments cells were considered as labelled if they had more than 3 grains over the nucleus The background labelling never exceeded 0 5 grains/100 μ^2 in counted areas All counts were done under oil immersion with a magnification on 1000

Definitions and Calculations

Howard & Pelc (1953) introduced the concept of the cell cycle M and S stand for mitosis and DNA synthesis, respectively, while G_1 and G_2 are time intervals during which no DNA synthesis occurs, although RNA and protein synthesis occur throughout both time intervals and the S phase (*Baserga* 1968) For practical purposes it is assumed in the following that the duration of mitosis is short and have to be divided equally between the G_1 and G_2 phases, i e when T_M is mean transit time of mitosis and transit time is the time elapsed between entering and leaving a compartment, we have

T_{G1} mean transit time of $G_1 + \frac{1}{2}$ M
T_{G2} mean transit time of $G_2 + \frac{1}{2}$ M
T_S mean transit time of S
T_C mean transit time of the cell cycle =
$T_{G1} + T_S + T_{G2}$,
SDT_{G1}, SDT_{G2}, SDT_S and SDT_C are the corresponding standard deviations

Doubling time T_D is the time it takes for the cells to double their number

Growth fraction G_F is the fraction of cells which are proliferating

$T_C = T_D$ when all cells are proliferating without cell loss.

By exposing cells to ^{3}H Tdr (a DNA precursor) for a brief period only those cells which are in the S phase incorporate ^{3}H Tdr and they are then distinguished as labelled by autoradiography

Labelling index LI is the fraction of cells synthesizing DNA

By determining the time course of percentage labelled mitoses a cyclic curve (PLM-curve) can be obtained Mean values and standard deviations of transit times in the different phases can be measured on this curve (*Quastler & Sherman* 1959, *Baserga* 1965, *Takahashi* 1966, *Cleaver* 1967) The distance between the peaks of two waves represents the mean cell cycle time T_C T_{G2} corresponds to the value between injection and the first 50 per cent intercept The minimum G_2 corresponds to the time between injection and the first appearance of labelled mitoses and maximum

G_2 to 100 per cent labelled mitoses T_S corresponds to the interval between the 50 per cent intercept on the rise and fall of the initial mitotic wave

T_{G1} is calculated from

$$T_C = T_{G1} + T_{G2} + T_S$$
$$T_{G1} = T_C - (T_{G2} + T_S)$$

It can be proved that the slope at the 50 per cent points on the ascending and descending part of the first wave of the PLM curve is inversely proportional to SDT_G and $SD(T_{G2} + T_S)$ respectively (*Takahashi* 1966) For practical purposes the interval on the abscissa between the 32 per cent and 68 per cent intercepts of the ascending part of the first wave represents SDT_{G2} The interval on the abscissa between the 68 per cent and 32 per cent intercept of the descending part of the first wave represents $SD(T_{G2} + T_S)$ SDT_S has been calculated as follows

$$SDT_S = \left[(SD(T_{G2} + T_S))^2 - (SDT_{G2})^2\right]^{1/2} \qquad (1)$$

T_M is calculated from

$$\text{Mitotic index} = \frac{T_M \ln 2}{T_C} \quad (\textit{Smith \& Dendy } 1962) \qquad (2)$$

T_D is determined in our investigation by cell counting It can also be determined from the PLM curve where

$$T_D = T_C = T_{G1} + T_{G2} + T_S \qquad (3)$$

when all cells in the system are proliferating and there is no cell loss

T_D can be calculated as follows

$$LI = \left(\exp\frac{T_S}{T_D}\ln 2 - 1\right)\exp\frac{T_{G2}}{T_D}\ln 2 \quad (\textit{Cleaver } 1965) \qquad (4)$$

where LI is labelling index shortly after a pulse labelling

RESULTS

Determination of Host Life Span

The relationship between the size of the leukemic cell inoculum i p and the host life-span was determined in groups of 20 mice after inoculation with 10 to 10^6 cells In Fig 1 it can be seen that the decrease in the average length of host life-span between each consecutive tenfold increase in size of leukemic cell inoculum is 1 6 days Since a tenfold increase in cell number represented 3 25 doublings of the population, the average T_D could be calculated by the formula

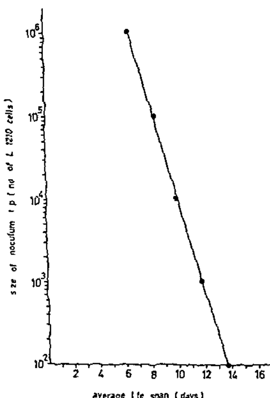


Fig 1 Relationship between size of inoculum i p (no of L 1210 cells) and untreated host life-span For every inoculum 20 mice were used

$$\text{average } T_D = \frac{1\ 6 \text{ days}}{3\ 25} = 0\ 49 \text{ days}$$

A detailed determination of host life-span after 10^5 cells is shown in Fig 2 It was seen that the first mice died after 6 days and none survived 13 days after transplantation Between day 7 and 8, 55 per cent of all mice included in the experiments died Average host life span was calculated as 7 8 days. The lowest average host life span in one experiment was 7 2 days, the highest 8 5 days

Determination of Cell Population Doubling Time

By counting the number of tumour cells in the peritoneal cavity at different times after transplantation it was possible to calculate the doubling time of the ascites tumour Cell counts were made from day 4 to 9 after

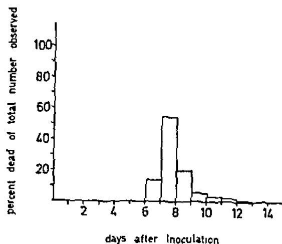


Fig 2 Host life span data for untreated mice after 10^5 L 1210 cells i p Data from 18 experiments with a total of 339 mice

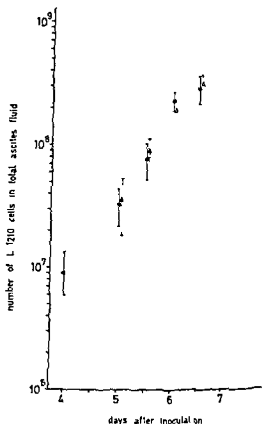


Fig 4 Influence of ^3H Tdr on growth of L 1210 ascites tumour Relationship between number of L 1210 cells in total ascites fluid and time after inoculation of 10^5 cells i p All determinations represent average of 10 mice Vectors represent standard error of the mean ● 20 µCi ^3H Tdr on day 4 after transplantation △ controls

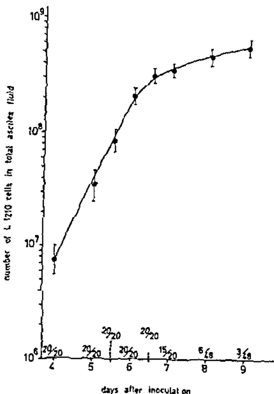


Fig 3 Relationship between number of L 1210 cells in total ascites fluid and time after inoculation of 10^5 cells i p The numbers along the abscissa indicate the number of mice surviving at the time of cell counting over the total number randomized to the eight groups Data from 2 experiments with a total of 216 mice Vectors represent standard error of the mean

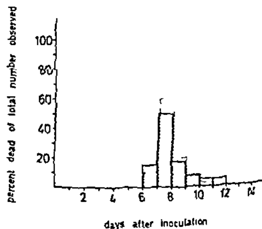


Fig 5 Influence of ^3H Tdr on life span of mice after transplantation with 10^5 L 1210 cells i p Dotted line 20 µCi ^3H Tdr day 4 Solid line controls Each group consisted of 40 mice

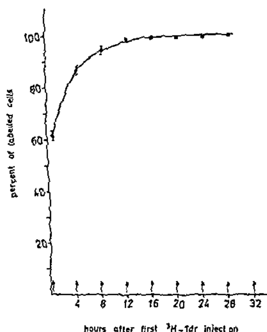


Fig 6 Continuous labelling of 6 day old tumour after 5 µCi ³H Tdr every 4 hour for 28 hours *Arrows indicate ³H Tdr injections* Vectors represent standard error of the mean

transplantation In Fig 3 the average cell count on a logarithmic scale is shown as a function of time

The cell growth was exponential from day 4 to 6 with a doubling time of 10 1 hours (calculated according to the method of least squares) A gradual prolongation then occurred and T_D was about 25 hours between day 6 and 7, and about 75 hours from day 7 to 9 (T_D calculated from the tangent to the growth curve)

Effect of ³H-Tdr on Doubling Time and Host Life-Span

On day 4 following transplantation, 40 mice were given 20 µCi ³H-Tdr i p, 50 mice given 0 2 ml NaCl i p served as controls Ascitic fluid cell counts were performed from day 4 to 6½ in 10 controls and 10 mice given ³H Tdr (Fig 4) The doubling times from day 4 to 6 were found to be identical, about 10 hours in the two groups Regression analysis (*Hald* 1952) using day 4 as the start value showed no significant difference between the two curves

Two additionally groups of 40 mice were treated in the same way and host life-span observed Average survival was 7 8 and 7 9 days, respectively, range 7–11 and 7 12 days A Chi square test showed no significant difference between the groups Data for the two groups are shown in Fig 5

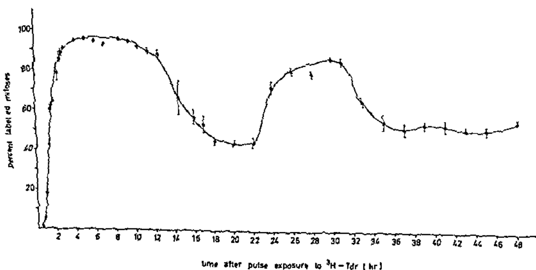


Fig 7 The cell cycle of L 1210 ascites tumour determined by per cent labelled mitoses All mice *received* 5 µCi ³H Tdr on day 6 of tumour *growth* Vectors represent standard error of the mean

TABLE 1 *Cell Cycle Data Derived from the Per Cent Labelled Mitoses Curve*

T_C	22.0	hours	
T_{G1}	5.6	–	
T_S	15.0 ± 1.8*	–	
T_{G2}	1.4 ± 0.7*	–	
T_M	0.3 ± 0.04§	–	Calculated from Mitotic Index $= T_M \frac{\ln 2}{T_C}$
T_D	22.0	–	Calculated from LI $= (\exp \frac{T_S}{T_D} \ln 2 - 1) \exp \frac{T_{G2}}{T_D} \ln 2)$

* *Represents standard deviation*
§ Represents standard error of the mean

Determination of Growth Fraction

A continuous labelling experiment was carried out in 48 mice on day 6 after transplantation by administration of 5 μCi ^{3}H-Tdr i p every 4 hours for 28 hours and autoradiographs were prepared from six animals one hour after every injection and the percentage of labelled cells scored (Fig 6) The start labelling index was 61 5 with range from 56 0 per cent to 66 0 per cent The standard error of the mean, which are all shown in the figure, was always below 1 6 per cent

After 13 hours and 5 ^{3}H-Tdr injections, more than 98 per cent of the tumour cells were labelled This corresponded to a growth fraction very close to 1 0

Mitotic index determined from 10 tumours 6 days after transplantation was found to be 1 08 per cent with a standard error of *the mean of 0 04 per cent*

Determination of Cell Cycle Time by Labelled Mitoses

70 mice were given *5 μCi ^{3}H Tdr* on day 6 following transplantation The next 48 hours, ascitic fluid was withdrawn from 5–7 mice at short intervals and prepared for autoradiography and the percentage of labelled mitoses scored Fig 7 shows the PLM curve with two waves of labelled mitoses The first wave *is asymmetrical, the second* not so well defined as the first one

Table 1 shows T_C, T_{G1}, T_{G2} and T_S determined from the PLM curve SDT_{G2} and SDT_S were calculated from formula 1 From the determined mitotic index, T_M (time spent in metaphase and anaphase) was calculated as 0 3 hours (formula 2) and from the experimentally determined LI and mean cell cycle phases, T_D was calculated according to formula 4 as 22 hours

DISCUSSION

The simplest way of characterizing the proliferation kinetics of a tumour is direct measurement of the tumour doubling time T_D This method is quite exact in ascites tumours, where the total number of cells can be counted directly, except when the cell counts are low

Using this method T_D was determined in *our L 1210 system as 10 hours from* day 4 to *6, then a gradual prolongation* occurred to about 25 hours between day 6 and 7, and to about 75 hours from day 7 to 9 The growth curve suggested that with a cell population less than 10^9 cells the growth was exponential

Another way of determining T_D is the host life-span method described by *Skipper et al* (1964) It gave an average T_D of 0 49 days (11 7 hours), which was in good *agreement* with the T_D determined on day *4 to 6* by cell counting on the exponential *part of* the growth curve

The *shape of the* growth curve—an *initial* exponential phase until day 6 followed by a continuous decline in the growth rate—can within the limits of the standard *error be*

fitted equally well to a Gompertz function (*Laird* 1964, 1965) The decelleration in the growth rate associated with the increase in tumour mass is a well known phenomenon in ascites tumours (*Lala & Patt* 1966, *Frindel et al* 1969, *Peel & Fletcher* 1969) It may result from a decrease in the growth fraction, a prolongation of the cell cycle or an increasing cell loss either by cell death or migration from the relatively closed limits of the abdominal cavity

In order to clarify these problems and in order to further characterize the cell cycle of the tumour cells it was necessary to use ³H Tdr labelling methods Control experiments were then necessary to examine the influence of the isotope itself In our system it was not possible to show either alterations in T_D from 1 hour to 2½ day after 1 0 μCi ³H Tdr per gram mouse, or alterations in host life span after the same dose on the same day after transplantation Contrary to this found *Lisco et al* (1961) has shown that ³H Tdr 10 0 μCi per gram mouse given 40 hours after transplantation of 2 × 10⁶ Ehrlich ascites tumour cells was able to diminish significantly the number of EAT cells on day 5 8 and 12 after transplantation, in per cent of controls given the same amount of cold thymidine However in his experiments the ³H Tdr dose was ten times higher than ours, and it was divided into 6 injections covering a span of 20 hours, causing a 100 per cent labelling of the tumour cells, while our pulse dose only labelled 60 per cent With a dose of 1 0 μCi per gram mouse *Lisco* only found an insignificant reduction in the number of tumour cells Also using high doses of ³H Tdr from 2 0 μCi and up, *Post & Hoffman* (1965, 1967) were able to show alterations in cell cycle time of hepatocytes in young rats 3–5 weeks later Thus the lack of changes in T_D or in mean survival in our system could be explained from a lower total radiation dose per cell, due to the use of a smaller amount of ³H Tdr per gram animal and a shorter exposure time of the cells to the isotope

The growth fraction determined by continuous labelling for 28 hours on day 6 after transplantation was very near 1, indicating that the whole tumour cell population was proliferating, at this time T_C was found from the PLM curve to be 22 hours The agreement between a T_D of 25 hours determined between day 6 and 7 and a T_C from day 6 of 22 hours, together with a growth fraction of 1 indicated a small or negligable cell loss (*Steel* 1967) This was in agreement with a finding by *Hofer & Hofer* (1971), using ¹²⁵IUDR labelled L 1210 cells, which showed that cell death among peritoneal L 1210 cells was very low (less than 5 per cent per day) and did not significantly influence the overall growth of L 1210 ascites populations

It was therefore most likely that the decrease in growth rate was due to a prolongation of T_C with the age of the tumour It then became important to define the part of the growth curve at which cell cycle characterisation was performed

By cell counting *Skipper et al* (1964) determined a T_D of 13 hours, while *Yankee et al's data* (1967) showed a T_D of 14 5 hours, both calculated from the exponential part of the growth curve From the same part of the growth curve we found T_D to be 10 hours

The shorter T_D in our tumour was in accordance with the shorter survival time of the mice after our standard inoculum of tumour cells *Skipper et al* (1964), in his extensive data, has published a T_D of 13 2 hours using the host life span method with a survival time ranging from 8 2–9 0 days after 10 cells i p, with the same technic and the same inoculum we found T_D to 11 7 hours and a mean survival of 7 8 days

This also meant that the plateau on the growth curve was reached sooner and on day 6 when the PLM experiments were performed, we were therefore closer to the plateau than the above mentioned investigators This explains why our T_C of 22 hours determined on day 6 is longer than that determined by others *Yankee et al* (1967) found T_C to be 11 8 hours, *Wheeler et al* (1967) 15 7 hours and *Skipper* (1968) 14 5 hours, all determinations made on the same day of

tumour growth and with the same inoculum of tumour cells

The discrepancy between our T_C and T_D and the values found by the authors above mentioned, shows that it is not possible directly to use the information obtained from other L 1210 sublines in other types of DBA/2 F_1 hybrid mice, in spite of a similar technic

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The author is indebted to S Olesen Larsen for assistance with statistical evaluation The excellent technical assistance of Mrs *E Steensgård* and Mrs *L Stein* is gratefully acknowledged

The work was supported with grants from The Danish Medical Research Council, The Daell Foundation and *The Hasselbalch Foundation*

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THE SENSITIVITY OF THE SKIN OF HAIRLESS MICE TO THE TUMORIGENIC ACTION OF COAL TAR IN RELATION TO THE TETRAZOLIUM TEST

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A 1 per cent solution of crude coal tar from the Norwegian Coke Factory was tested with the tetrazolium test for carcinogenicity The result was as expected positive The carcinogenic potency of this tar in long term paintings on hairless mouse skin confirmed the well known carcinogenicity of coal tar, but revealed also that this particular strain of hairless mice is rather sensitive to chemical carcinogens

The tetrazolium test (*Iversen* 1963) for carcinogenicity is based on the abnormally high amount of formazan deposited in carcinogen treated epidermal cells incubated with a tetrazolium salt In 1968 our Institute received for carcinogenicity testing some coal tar from the Norwegian Coke Factory We were asked to measure the carcinogenic potency of this particular tar by the tetrazolium test, and by studying the effect of long term application on hairless mouse skin

MATERIALS AND METHODS

Animals

Hairless (hr/hr) mice were used in the experiments Spontaneous skin tumours have not been observed in these animals At the beginning of each experiment the animals were 70–80 days old and thus in a resting period of hair growth The animals were housed in plastic cages 5 in each cage, and fed a standard diet and water *ad libitum* All animals were kept in the same room The cages were cleaned and new food and fresh water supplied about noon each day

The Tetrazolium Test

A 1 per cent solution of coal tar in benzene was applied to the mouse skin and the tetrazolium test was performed as described in detail elsewhere (*Iversen* 1963)

Tumour Induction Experiment

Fifty mice were randomly selected and divided into two groups of 25 males and 25 females respectively The coal tar was provided by Norsk Koksverk A/S, and is ordinary coal tar from the coke production in that factory The coal comes from the Norwegian Coal Mines at Spitsbergen The back skin of the mice were painted with a 1 per cent solution of tar in benzene twice weekly with a fine brush during 13 months The animals were observed weekly for skin tumours An outgrowth was considered a papilloma when it was at least 1 × 1 mm Each papilloma present after two or more examinations was registered as a

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tumour An attempt was made to differentiate clinically between papillomas and carcinomas as soon as they developed by assessment of degree of infiltration as judged by palpation All animals were kept until death or killed earlier because of deterioration due to the malignant tumour or other disease Whenever possible, a necropsy was done and at least one of the largest skin tumours from every animal was examined histologically All lesions registered as carcinomas were eventually verified histologically Infiltration below the musculus panniculus was used as criterion of malignancy for the carcinomas The occurrence of reticuloses or other sarcomas in internal organs was not recorded

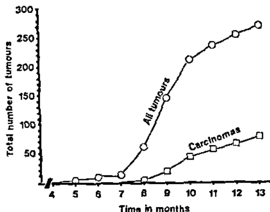


Fig 2 Cumulative increase in total number of tumours and of carcinomas, respectively during 13 months paintings with a 1 per cent solution of coal tar in benzene

RESULTS

The Tetrazolium Test

The test was performed twice The results were 1,21 and 1,51, respectively, which means that a 1 per cent solution of coal tar in benzene gave a positive result (*Iversen* 1963)

Number of Tumour Bearing Animals

49 animals survived until the first papilloma appeared The results are presented according to the traditional method, *viz* tumour yield in relation to number of animals alive at the appearance of the first papilloma The percentage of tumour bearing animals with respect to time is shown in Fig 1 After 4 months the animals started to get tumours,

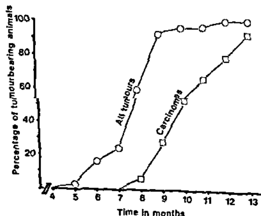


Fig 1 Percentage of tumour bearing and carcinoma bearing animals respectively, during 13 months paintings with a 1 per cent solution of coal tar in benzene

and beween the 6th and the 9th month the number of tumour bearing animals increased rapidly After 12 months all animals had tumours The carcinomas started to appear at 8 months There was more than 90 per cent carcinoma bearing animals at 13 months

Total Number of Tumours

The cumulative tumour incidence with respect to time is plotted in Fig 2 The total number of tumours increased rapidly after the 7th month, and the total number of carcinomas increased from the 8th month, and at about a steady rate At the 13th month the mean number of papillomas per animal alive at the appearance of the first tumour was 5,4, and the corresponding value for carcinomas was 1,6

DISCUSSION

Based on the hypothesis that the presence of hair follicles plays a role in chemical carcinogenesis of mouse skin, *Giovanella et al* (1970) compared the yield of skin tumours in their strain of hairless mice and in mice of the same strain with hair, using the induction promotion technique They concluded that the skin of hairless mice was relatively refractory to chemical carcinogenesis Hairlessness is, however, a recessive genetic char-

acteristic that can be introduced into different strains Other families of hairless mice may be more tumour sensitive The results of this study shows that the hr/hr strain of hairless mice kept at this Institute must be characterized as a sensitive one A direct experiment comparing the relative sensitivity of hairless mice and mice with hair of the same strain is in progress

The tetrazolium test has been clearly positive for all strong carcinogenic hydrocarbons hitherto tested and for many other skin carcinogens It is however not completely specific and is often giving borderline values for weak carcinogens The positive result of the tetrazolium test performed with a 1 per cent solution of crude coal tar in benzene corresponds well with the carcinogenic potency of the same solution of this tar, as demonstrated in long term skin paintings

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Acta path microbiol scand Section A 80, 615–620, 1972

THE ADRENAL GLANDS IN CYSTIC FIBROSIS

Morphology Correlated with Clinical Findings

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The findings obtained by morphological investigation of the adrenal glands from 23 patients with cystic fibrosis (CF), have been compared with the findings in 46 controls In 14 patients with CF, changes in the form of necrosis and/or haemorrhage in the permanent cortex were found and, in addition, in 3 of these there was an accumulation of lipid The changes were independent of age In 6 control patients, similar changes localized to the same site were found, but almost exclusively in older children No correlation was found between these changes and the clinical course of the disease A number of different investigations suggesting changes in the steroid metabolism in CF are reported

Cystic fibrosis (CF) is an inherited disorder transmitted as an autosomal recessive trait, the aetiology of which is still unknown It is a generalized disease involving changes in almost all organs Anatomical defects have been found in the male genitals (14, 13), and there is a sex difference in the courses and prognosis of the disease (18) As changes have also been found in the steroid metabolism of these children, it was considered that an investigation of the morphology of the adrenal glands in CF might be of some value

MATERIAL AND METHODS

The material comprises the adrenals from 23 patients with CF, together with the adrenals from 46 control patients who died from pulmonary, cardiac neurological and certain other diseases, none of which were considered to have any specific influence on the morphology of the adrenal glands None of the control patients had any signs of CF at autopsy, in particular no microscopic changes in the pancreas were observed The 23 patients with CF died during the period 1952–1971, and included 13 boys and 10 girls Twelve came to autopsy at the Pathological Institute of Rigshospitalet, 11 at other hospitals

Table 1 shows the ages of the patients in the two groups At autopsy, all patients with CF showed typical changes in the pancreas and the lungs In one case, however, the pancreas was not examined histologically, but this patient had typical gastro intestinal symptoms, typical histological changes in the lungs and liver and, in addition, two siblings had CF The diagnosis must thus be regarded as certain for all patients, although the sweat electrolytes had been examined only in 13, the duodenal fluid only in 8 Nine patients had one or more siblings with CF Six patients had received adrenal cortical hormone during the last days, two patients had received ACTH, or ACTH combined with cortisone, during a longer period, and one patient had received methandrostenolone for a period of 5 months before death None of the patients in the control group had received hormone treatment

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TABLE 1 *Age Distribution of Patients with Cystic Fibrosis and Controls*

Age	3–7 days	8–30 days	1–12 months	1–8 years	9–30 years	Total
Cystic fibrosis	2	3	7	5	6	23
Controls	10	12	10	7	7	46

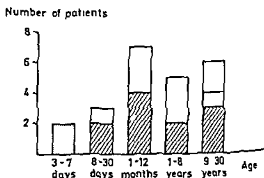
The adrenal glands were fixed in formalin, embedded in paraffin, and stained with haematoxylin eosin and van Gieson supplemented in a few cases by Sudan III Each preparation was examined for width of the various cortical zones which were measured by means of a measuring ocular in arbitrary units and compared to the width of the entire adrenal cortex Each separate zone was examined for morphological changes and the following changes were recorded destroyed cell membrane and nuclear pyknosis in large areas were regarded as necrosis, and areas with the individual cells surrounded by erythrocytes were regarded as regions of haemorrhage Foci consisting of large, bright swollen cells with sudanophilic material were recorded as accumulations of lipid In addition, the width of the medullary zone was evaluated in relation to the cross section of the adrenal gland as well as its extent in the long axis of the adrenal, by examination of each separate section On the basis of these evaluations three grades were established as a measure of the development of the medullary zone Inflammatory foci have not been included in this investigation

RESULTS

The involution of the foetal cortex showed the same morphological characteristics in the CF-group and in the control material In both groups, this process had terminated by the end of the first year of life in agreement with the results obtained in a larger series already described (1, 2)

Differentiation of the permanent cortex into the three well-known zones likewise showed the same features in the two groups The zona glomerulosa could in all cases be identified around the age of 6 months, and was found to be well-developed by the end of the first year of life The zona fasciculata could be recognized already from the time of birth, while the zona reticularis did not occur constantly until the age of 1 year

The medullary zone was found to be only slightly developed during the first weeks of life in both groups In patients with CF, it was well-developed already around the first month, while a corresponding degree of development in the control material was not



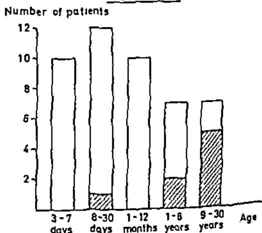


Fig 1 Changes in the permanent adrenal cortex in patients with cystic fibrosis and controls

found until the infants were 6 months old or more

The CF-material included 14 cases with necrosis and/or haemorrhage localized to the zona glomerulosa, the zona fasciculata or the transition between these, while only 8 of the control patients presented similar changes These control patients had died from the following causes pneumonia, cardiac malformation, cirrhosis of the liver, traffic accident, neurological disease (3 patients), and unknown cause None of these cases showed any signs of CF As shown in Figure 1, changes in the adrenals were found in the control group almost exclusively in patients in the two oldest age groups, while there was a uniform distribution over all ages in the CF-group In addition, in 3 of the 14 CF-patients with the above-mentioned changes, accumulation of lipid was found in the transitional zone between the zona glomerulosa and the zona fasciculata (Fig 2) These 3 patients had died at the ages of 4 days, 7 days and 23 years, respectively. Finally, a large calcification was found in the adrenal cortex in a single case No accumulation of lipid was found in the control group The χ^2-test showed that the number of patients with adrenal changes in the CF-group differed significantly from that in the control material ($\chi^2 = 13.5$, $p < 0.001$)

No clinical differences between the 14 CF-patients with changes in the adrenal cortex and the 9 CF-patients without these changes were found The age and the duration of symptoms were the same in both groups There was no difference in the time of onset of the pulmonary and gastro-in-



Lipid accumulation in the transitional zone between the zona glomerulosa and the zona fasciculata HE × 100

testinal symptoms, and both groups comprised patients who had been operated on for meconium ileus, although 4 of 5 patients with this complication showed changes in the adrenal cortex. The distribution of boys and girls was the same, just as was the height and weight retardation of the patients and the occurrence of CF among siblings. Among the seven patients on whom bacteriological autopsy was performed, 4 showed growth from several organs, all of these 4 had changes in the adrenals. Three patients showed no growth, and 2 of these had changes in the adrenals. None of the patients had any clinical tendency to haemorrhage, but the serum prothrombin had been determined in nine. Among four patients with a prothrombin value less than 50 per cent, two had haemorrhage in the adrenal cortex, and among five patients with a prothrombin value above this, one had haemorrhage in the adrenal cortex. At autopsy, eight patients had cirrhotic changes in the liver. Seven of these had focal biliary cirrhosis (19), and five out of these seven had necrosis and/or haemorrhage in the adrenal cortex. One patient had multilobular biliary cirrhosis (19), the same patient had haemorrhage in the adrenal cortex and was the sole patient with splenomegaly. Among the six patients who received hormone treatment shortly before death, four had changes in the adrenal cortex, and one of the two who had received this treatment for a longer period had changes in the adrenal cortex. None of *these patients who had received hormone* treatment, however, showed any accumulation of lipid in the adrenal cortex. The patient who had been treated with methandrostenolone showed small necroses and accumulation of lipid.

DISCUSSION

Our findings by histological examination of the adrenal glands from patients with CF showed that the medullary zone had a tendency to develop at an earlier stage in these than in the controls, while there was no difference either with regard to the involution of the foetal cortex or the differentiation of the permanent cortex. Independent of age these patients showed an increased incidence of unspecific changes in the adrenal cortex in the form of necrosis and haemorrhage in the zona glomerulosa and the peripheral part of the zona fasciculata. Such changes have been demonstrated previously in severe infectious deseases (6), and the chronic lung infection in CF may possibly be a contributory factor, just as in CF there may be a tendency to haemorrhage on account of *malabsorption, liver involvement and hyper*splenism. The extent to which these conditions have played a role in the present series could not be established. Conditions of chronic stress usually cause a reduction in the fat content of the adrenal glands (6), whereas an accumulation of lipid was found in the adrenal cortex of 3 patients *in the* present material, two of whom, however, were only 4 days and 8 days old. It may be added that in a larger series of 782 stillbirths and newborn infants we have found 7 cases with corresponding changes and thus lipid accumulation localized to this site may be assumed to be relatively rare in an autopsy material of newborn. (Among the 7 patients mentioned, one had CF and is included in the present series, while the others died from various other causes and showed no signs of CF at autopsy.)

Only few investigations of the morphology of the adrenal glands in cases of *CF* are *available. Bergstrand (3) has* examined the adrenal glands from a patient aged 7 weeks and found aberrant adrenal cortex tissue consisting of cells larger and more eosinophilic than those in the actual adrenal cortex as well as small haemorrhages in the medulla. *Lanman* (15) found normal involution of the foetal cortex in three patients aged 6 to 14 months. *Bongiovanni et al* (4) examined the adrenal glands from six patients aged 4½ years up to 17 years and found in all a hyperplastic adrenal medulla filled with chromaffin positive tissue and many cells with nuclei larger than normal

Chodos et al (5) found a lipid laden adrenal cortex in a patient aged 10 years *Garrido* (10) has collected a series of 12 CF patients aged 1 day up to 7 months and found that nine of these had a zona glomerulosa which was either undeveloped or difficult to recognize. However it is not until an infant is 6 months old or more that we find that the zona glomerulosa occurs constantly and can be recognized with certainty both in the CF group and in the control series and we regard this as the normal course of development

A few investigations on the hormones of the adrenal glands and their function in these patients are also available *Bongiovanni et al* (4) supplemented their histological investigation with a biochemical study of the adrenal tissue from six patients and found in five an elevated content of catecholamines while the content of cortisol 11 desoxycortisol and 17 ketosteroids did not deviate from normal *Siegenthaler et al* (20) found normal aldosterone excretion in four patients *Chodos et al* (5) found a normal excretion of 17 hydroxy and 17 ketosteroids in 27 patients as well as a normal concentration in plasma of 17 hydroxysteroids cortisol and corticosterone before and after ACTH stimulation while the half life of cortisol was prolonged in patients who were 1 to 6 years old *Grand et al* (11) demonstrated in 11 patients apart from a few showing an abnormal water load test that the adrenal function was normal if evaluated by the glucose tolerance test the excretion of 17 hydroxysteroids and 17 ketosteroids as well as the excretion of 17 hydroxysteroids after stimulation with ACTH and metyrapone Further *Montalvo et al* (16) found in a series of six CF patients including also their parents that only the most severely affected patient had an increased aldosterone secretion rate aldosterone metabolic clearence rate and plasma aldosterone while the others showed normal conditions In contrast to findings by others *Gray & Hamilton* (12) have demonstrated a reduced excretion of 17 hydroxysteroids and a strongly reduced excretion of dehydroepiandrosterone sulphate *Flensborg & Johnsen* (9) have found a reduced excretion of 11 hydroxyetiocholanolone and 11 ketoetiocholanolone while the excretion of androsterone etiocholanolone dehydroepiandrosterone 11 ketoandrosterone and 11 hydroxyandrosterone was found to be normal

Also other investigations are available which point in the direction of hormonal changes in CF The congenital anatomic defects in the derivatives from the Wolffian duct (14 13) suggest the possibility of a hormonal defect even during foetal life The late development of puberty and the excess mortality of girls by comparison with boys particularly after puberty (18) also suggest endocrine involvement in CF just as does the often strongly retarded bone age found to be most pronounced in girls (17) Other findings which point in this direction are the demonstration of parallelism between the changes in the sodium/potassium ratio in the sweat in normal newborn infants and the involution of the foetal adrenal cortex (7) as well as the fact that the sodium content of the sweat and the sodium/potassium ratio tend to become normal on administration of methandrostenolone (8)

The picture of the morphology and function of the adrenal glands in CF is thus a very variegated one and their possible role in the pathogenesis of the disease is still unknown The various investigations which have been made are not conclusive but suggest changes in the steroid metabolism and our findings would appear to support this view

This study was granted by *The Michaelsen Fund* the *Danish insurance companies "National* and *Haand i Haand"* and The *Danish Cystic Fibrosis Research Foundation*

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Thymectomized animals treated with saline (35 animals)

The 5 FUdR was dissolved in 0 9 per cent saline and administered intraperitoneally in a single dose of 100 mg per kg body weight The control animals were injected with 0 5 ml 0 9 per cent saline

The guinea pigs were investigated 2, 4 and 8 days after the treatment and thus, 16, 18 and 22 days after the operation At investigation the body weight had increased to 260–350 g The animals were anaesthetized with 2 5 per cent Nembutal sodium (25–50 mg per kg b w) An incision was made over the spleen and blood samples were taken from one of the splenic veins and the splenic artery (for technical details, see *Ernstrom* & *Sandberg* 1968)

The blood samples were used for white cell counts in a haemocytometer with differentiation between polynuclear and mononuclear cells, and for differentiation of lymphocytes into subclasses including cells with different mitochondrial content This differentiation was made in preparations stained supravitally with Janus green B and neutral red (for details, see *Ernstrom et al* 1969) Lymphocytes with 0–15 mitochondria and with more than 15 mitochondria are denoted as small and large lymphocytes, respectively, as the mitochondrial content of the lymphocyte is correlated to the size of the lymphocyte (*Wiseman* 1931, *Fichtelius* & *Larsson* 1961, *Ernstrom* & *Larsson* 1963)

The number of lymphocytes and granulocytes per µl of blood from the splenic vein and artery was calculated The splenic veno-arterial difference in the number of different white blood cells was obtained from each animal and the differences were analysed statistically by Student s *t* test. The animals were sacrificed and the spleen, cervical and mesenterial lymph nodes were dissected and weighed Differences in organ weights were studied using Student s *t* test

RESULTS

Sham-Operated Animals

A release of lymphocytes from the spleen was demonstrated by a veno arterial difference in content of cells in blood from the splenic vein and the splenic artery (Table 1) This difference was highly significant for both small and large lymphocytes ($p<0\ 001$)

Thymectomized Animals

The weights of spleen and lymph nodes were not significantly changed by the thymectomy (Fig 1) The blood granulocytes were also unchanged (Fig 2) The blood lymphocytes were decreased in number ($p<0\ 01$, Fig 2)

A highly significant splenic release of lymphocytes consisting of both small and large lymphocytes were found, although quantitatively, the veno-arterial differences were smaller than in the sham operated animals (Table 1)

5-FUdR-Treated Sham Operated Animals

The cervical lymph nodes decreased in weight after treatment with 5 FUdR ($p<0\ 01$), but had returned to normal weight after 4 days The weights of the spleen and the mesenterial lymph nodes were not significantly changed (Fig 1) The blood granulocytes were unchanged The lymphocytes were markedly decreased in number after 2 and 4 days ($p<0\ 001$) After 8 days the number had returned to normal again (Fig 2) The recovery of the large lymphocytes was more rapid than that of the small lymphocytes (Fig 3)

The splenic release of lymphocytes was decreased, although it was still significantly demonstrable (Table 1) The decrease was significant for the small lymphocytes and most pronounced 2 days after 5 FUdR treatment (p value for the decrease $<0\ 05$)

5-FUdR Treated Thymectomized Animals

The decrease in organ weights due to 5 FUdR treatment was not significantly different from that in the 5 FUdR-treated sham operated animals However, after 8 days, the weights of lymph nodes and spleen tended to be lower in the thymectomized animals than in the corresponding sham operated animals (Fig 1) The blood granulocytes were not significantly changed in number (Fig 2) The blood lymphocytes were decreased in number after 2 days ($p<0\ 01$) and then increased above the values of their saline-treated controls (Fig 2) This increase was attributed to the large lymphocytes (Fig 3)

TABLE 1 *Splenic Veno Arterial Difference in Number of Lymphocytes per µl of Blood in Thymectomized and Sham Operated Guinea Pigs after a Single Injection of 5 FUdR in a Dose of 100 mg per kg Body Weight Mean ± Standard Deviation of the Mean*

Operation	Treatment	Days after 5 FUdR	No of animals	No of small lymphocytes	No of large lymphocytes
Sham-operated	saline		22	462±100†	1074±195†
Sham-operated	5 FUdR	2	13	138± 36§	805±164†
Sham-operated	5 FUdR	4	9	310±103*	1357±286§
Sham-operated	5 FUdR	8	5	259±129	483±249
Thymectomized	saline		35	367± 62†	753±113†
Thymectomized	5 FUdR	2	10	346±105*	702±221*
Thymectomized	5 FUdR	4	12	120± 56	923±196†
Thymectomized	5 FUdR	8	11	154±107	686±263*

*, §, † indicate $p<0.05$, $p<0.01$, $p<0.001$, respectively, for the veno-arterial difference

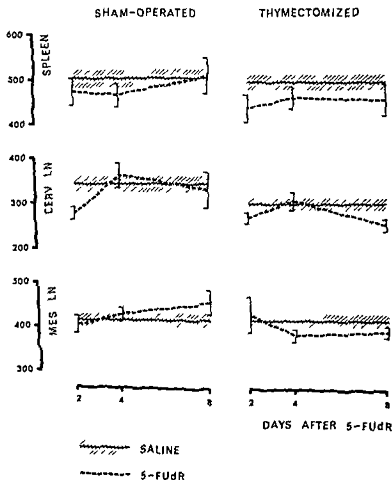


Fig 1 Weight (mg) of the spleen, cervical and mesenterial lymph nodes in sham-operated (left) and thymectomized (right) guinea pigs at different intervals after treatment with 5 FUdR The hatched area indicates the organ weights of control animals injected with saline Mean ± standard deviation of the mean

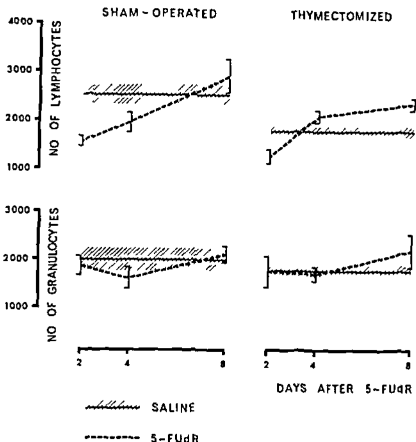


Fig 2 *Number of lymphocytes and granulocytes per µl of splenic artery blood in sham-operated (left)* and thymectomized (right) guinea pigs at different intervals after treatment with 5 FUdR The hatched area indicates the cell number in blood from control animals treated with saline Mean ± standard deviation of the mean

The splenic release of small lymphocytes was diminished to a minimum value 4 days *after treatment with 5 FUdR (p value for* the decrease <0 05) while the release of large lymphocytes was unchanged (Table 1)

DISCUSSION

The present investigation has confirmed the occurrence of a release of splenic lympho cytes to the blood in guinea pigs during the experimental circumstances described (see *Ernström* & *Sandberg* 1968 *Sandberg* 1970a) In the thymectomized animals this release was only slightly depressed This is in agreement with the report by Sandberg that thymectomy had little effect on splenic discharge of lymphocytes in unimmunized animals but abolished the increase in splenic release of lymphocytes seen in normal and *sham operated animals after immunization* with sheep red blood cells *(Sandberg* 1970b 1972)

The treatment with 5 FUdR reduced the number of circulating small blood lympho cytes both in sham operated and thymec tomized animals The small lymphocytes re turned to control values *8 days after 5 FUdR* irrespective of thymectomy The treatment with 5 FUdR also decreased the splenic dis charge of small lymphocytes and this release had not returned to normal values after 8 days neither in sham operated nor in thymectomized animals The decreased out put of splenic lymphocytes is most probably due to the inhibition of DNA synthesis and

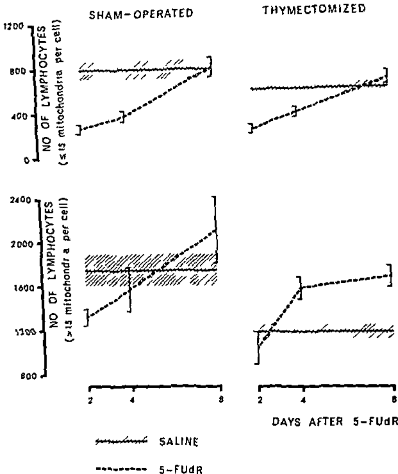


Fig 3 Number of lymphocytes per μl of splenic artery blood in sham-operated (left) and thymectomized (right) guinea pigs at different intervals after treatment with 5 FUdR The hatched area indicates values from control animals treated with saline The lymphocytes are subdivided into smaller cells (≤ 15 mitochondria per cell) and larger cells (> 15 mitochondria per cell)

cellular proliferation in the spleen caused by 5 FUdR The results indicate that the splenic discharge of small lymphocytes is at least partly dependent on an intact DNA synthesis

The curves for the number of large lymphocytes in the 5 FUdR treated animals thymectomized or not seem rather similar, although the baseline is lower in the thymectomized than in the sham-operated (Fig 3) Thus the results indicate that the splenic output of large lymphocytes and their recovery in the blood are independent of the actual presence of the thymus Despite the treatment with 5 FUdR the number of large lymphocytes tended to increase and the data are thus compatible with an attempt of this cell population to compensate for the effects of 5 FUdR on small lymphocytes in blood and lymphoid tissues The appearance of increased numbers of large lymphocytes in blood and lymphoid tissues is in fact a sign of regeneration and is demonstrated as early as 24 hrs after exogenous steroids irrespective of thymectomy (*Ernström* & *Larsson* 1967) and after irradiation (*Ernstrom* 1972) As the large lymphocytes are not thought of as a resting cell population their output and level might have been expected to be seriously affected by 5 FUdR, as this drug is

claimed to inhibit DNA synthesis and cell proliferation The experiment indicates that the effect of 5-FUdR is of short duration and overcome within 2 to 4 days

In previous investigations we have proposed that a burst in the release of lympho cytes from the thymus may be necessary for the rapid recovery of blood lymphocytes and spleen and lymph node weights to normal values after irradiation (*Ernström* 1972) or treatment with 5 FUdR (*Ernström & Nordlind* 1971) The present study in 5 FUdR-treated animals could not support this proposition, since blood lymphocytes returned to control values as rapidly in thymectomized as in sham-operated animals The weights of the spleen and cervical lymph nodes had a slower regeneration in the thymectomized animals, but the deviations were too small to be regarded as conclusive evidence of an important role for the thymus

As an indication of some effect of the thymus on lymphatic regeneration, the splenic release of small lymphocytes was more depressed in the thymectomized animals 4 and 8 days after treatment with 5-FUdR than in the sham operated animals (Table 1) Despite the fact that the deviations in organ weights and discharge of small lym phocytes from the spleen are small after combined thymectomy and 5 FUdR treat ment, it is still possible that they reflect a specific deficiency in thymus derived lympho cytes which could have functional consequences The importance of ultimately thymus-derived lymphocytes may still be great in the recovery from the effects of 5 FUdR treatment, provided that the effective cells have migrated from the thymus to periphery earlier than 16 to 22 days before the investigation

This investigation was supported by a grant from the Swedish Cancer Society

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Acta path microbiol scand Section A 80, 627-633, 1972

CEREBRAL MALFORMATIONS IN THE XYY SYNDROME

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The XYY syndrome predisposes to mental retardation, personality defects and neurological symptoms. In autosomal chromosome syndromes such features are often associated with cerebral malformations. There are, however, only a few reports on the pathology of the central nervous system in patients with sex chromosome aberrations, and so far, the neuropathology in the XYY syndrome has been entirely unknown. The present paper concerns a prepubertal mentally retarded boy with typical clinical and cytogenetic features of the XYY syndrome. The brain was megalencephalic with mild cortical dysplasia, diffuse neuronal heterotopias of white matter and signs of retarded cellular maturation. The observations allow no generalized conclusions concerning the role of an XYY sex chromosome constitution in the production of cerebral abnormalities but point to the importance of further studies in this field.

The phenotype associated with the XYY sex chromosome constitution is variable. Some cases are normally developed fertile males with normal intelligence (14, 29). In other cases there are abnormalities of the external genitalia and/or mental retardation (13).

Interest in the effect of an XYY sex-chromosome constitution was aroused by the finding of an increased incidence of this karyotype among mentally subnormal patients with aggressive behaviour and tall stature. No abnormal physical features distinguished these patients from normal men (22) but their personalities showed extreme emotional instability combined with an incapacity to tolerate even mild frustrations. They began their criminal activities at a very young age but there was no significant family history of crime or mental illness (23). There are only a few reports with detailed clinical descriptions of prepubertal patients with an XYY sex chromosome constitution (8, 17, 24) and to our knowledge there is no post mortem examination reported on any patient with the XYY syndrome clinically characterized by tallness in combination with mental subnormality and behavioural problems.

This report concerns the clinical features, cytogenetical data and autopsy findings in a boy with the XYY syndrome who suddenly and unexpectedly died at $11^{11}/_{12}$ years of age. Some data on this patient have been reported earlier (12).

CASE REPORT

Family history and clinical findings. At the time of the patient's birth his mother was 31 and his father 37 years of age. Both parents were normal and in good health. A male paternal first cousin of the patient was mentally subnormal and of tall

Received 7.iv.72

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Fig 1 The patient at 8½ years of age

stature The family history was otherwise negative The mother gave no history of abortions She had had two pregnancies, the first resulted in a normal girl whose subsequent development was normal The second pregnancy, resulting in the birth of the present patient, was uneventful He was born 2 weeks after term The delivery was normal The birth weight was 4250 g and height 56 cm The child's psychomotor development was somewhat retarded and he had obvious difficulties in keeping abreast at school He was abnormally sensitive, lacked initiative became very easily tired and had great difficulty in establishing contact with his schoolmates At periods he was easily angered but did not show any particular aggressive tendencies There appeared to be no predisposing family environment He has always been healthy

Physical examination at 8½ years of age showed a proportionally built boy with large somewhat low set ears (Fig 1) and short 5th fingers No other abnormalities were noted His height was 149 cm (about 10 cm above + 2 S D) His weight was 41 kg (normal for the height) The genitalia were normal The testes were of normal size and consistency The neurological examination was entirely within normal limits, except that the fine and coarse motor movements were clumsy He was passive, emotionally unstable and lacked self-confidence Using the Terman-Merrill and the Wechsler intelligence scale for children he fell largely in the 71 to 76 IQ equivalent ranges Routine tests on blood and urine were normal Protein bound iodine was 6 5 μg/100 ml serum Fasting growth hormone level 8 ng/100 ml plasma (normal for his age) The urinary excretion of follicle stimulating hormone, luteinising hormone, 17 ketosteroids, 17 hydroxysteroids, oestradiol, oestriol, oestrone, pregnanediol and pregnanetriol was normal Osseous development was slightly advanced for age The audiogram and the ophthalmoscopic and EEG examinations were normal

The patient has been subjected to repeated follow up examinations At $11^{3}/_{12}$ years his height was 165 5 cm, span 160 cm, weight 52 8 kg and head circumference 54 5 cm The external genitalia were normal The testes were of normal prepubertal consistency, measuring 3 × 2 cm

At $11^{11}/_{12}$ years of age he died suddenly and unexpectedly when he was walking home from school

Cytological studies A total of 110 metaphase plates from three independent blood cultures of the patient when he was 8½ years old showed 47 chromosomes including an extra chromosome similar to a Y chromosome Buccal mucosa cells lacked sex chromatin There were no drum sticks in 500 polymorph leucocytes examined Autoradiographic studies according to Schmid (26) showed that two chromosomes resembling a Y chromosome replicated late compared with the other small acrocentric chromosomes The parents of the patient had normal karyotypes

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Fig 2 Gross appearance of frontal lobe section showing an irregularly and partly abnormally wide cortex Appr × 5

Fig 3 Frontal cortex second lamina poorly outlined, containing few granular cells but in addition larger pyramidal nerve cells Cresyl violet, × 160

Fig 4 Temporal cortex the wide molecular layer (occupying upper half of picture) is too cellular, due to groups of undifferentiated cells The second, granular, layer has a normal appearance Cresyl violet, × 160

Fig 5 Centrum semiovale with diffuse heterotopia consisting of well developed neurons with satellite glial cells Cresyl violet, × 160

Fig 6 Lateral to the temporal ventricular horn there are subependymal rows and clusters of undifferentiated cells as well as mature neurons Cresyl violet, × 160

Fig 7 Capillary vessel in frontal hypothalamus surrounded by cuff of small dark round cells of an embryonal type Cresyl violet × 100


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When the patient was 11 *years old the quinacrine* mustard fluorescence pattern (6) as well as the autoradiographic pattern of the same chromosomes in cultured blood *cells was studied A total* of 69 cells were analysed A characteristic intense fluorescence pattern of the two late replicating Y chromosomes was *found These studies have been* reported in detail elsewhere (25)

Autopsy Findings

The autopsy showed a small pedunculated lipoma attached to the heart close to the left descending coronary artery Within the distribution area of the latter the myocardium showed ischaemic changes, probably caused by the lipoma In addition there were signs of aspiration

The brain, which weighed 1 710 grammes unfixed, showed no signs of oedematous swelling or increased intracranial pressure It appeared to have a simplified convolutional pattern particularly in *the frontal and central suprasylvian areas Here* the gyri were wider and straighter than normally

On frontally cut sections the callosal body was *found to be slightly thinned out, the ventricular* system was only insignificantly widened Though enlarged, the various structures appeared largely *normally shaped and proportionate The cerebral* cortex was wider than normally, particularly in the frontal and central areas mentioned above (*Fig* 2) *with the straight gyration*

On microscopical examination these cortical areas showed abnormalities of the following kind *The wide cortex was indistinctly laminated* In the normally granular frontal cortex the granular layers II and IV were faintly outlined and both *contained a heterogenous population of neurons,* some of which were large and triangular with a wide apical dendrite (Fig 3) Another feature *that contributed to the indistinctness of lamination* was a striking columnar arrangement of neurons, leaving wide "cellfree' spaces between the columns

The *border between the molecular layer and* the second cortical layer was often indistinct In the wide molecular layer there was an abnormal *number* of large nerve cells usually oriented close to and parallell with the pial limiting membrane Here also there were clusters of small round dark cells with little visible cytoplasm and a sharply outlined nucleus, with the appearance of glio- and neuroblasts (Fig 4) There was, how*ever, never* found a definite layer of such cells Such was also the picture in the molecular layer of the presubicular and enthorhinal cortex

The white matter, particularly that of the wide and "simple" gyri was studded with heterotopic large neurons, often surrounded by *satellite cells* (*Fig* 5) They were found deep down e g in the frontal paraventricular white matter, though they were most numerous further out towards the cor*tical plate* These changes were often bilaterally symmetrical and on staining for myelin showed up as a pale area with slightly reduced myelin *density*

The basal ganglia appeared essentially normal. In the frontal hypothalamus there were clusters of *small* dark round cells also seen around the inferior frontal and temporal horn of the ventricular system (Fig 6) Other similar cells formed cuffs around *hypothalamic capillaries below the frontal* horns (Fig 7) and appeared in the corresponding basal meninges Such changes did not occur outside this area There were no histiocytic or plasma cells and no scarring in connection with these changes Neither was there any sign of anoxic cerebral damage The ependymal lining was in places broken, with small *glial nodules projecting* into the ventricular lumen

Other organs were unremarkable, including their size, except for the testes These were of a normal size and appearance on cut sections Histologically there were areas of complete sperm production though *in other areas only less mature sperm stages* were seen The heads of the majority of sperms appeared abnormal In addition there was an obvious hyaline thickening of the tubular walls and in some areas there were only Sertoli cells and a lack of the germinal epithelium The interstitial cells appearrd unremarkable

DISCUSSION

The cause of the development of the mental *abnormalities found in our patient could* not be found in his childhood environment It seems thus possible that the XYY chromosome constitution is the main aetiological factor of his mental retardation and ab*normal personality development*

A key question is whether the reported cerebral malformations in our patient are related to the chromosomal anomaly

Among the autosomal syndromes, especially *trisomy 13 and 18 are reported to* show cerebral malformations such as arrhinencephaly, cerebellar hypoplasia and neuronal heterotopias as well as hypoplasia or agenesis of callosal body (*9, 19, 27, 28, 30*) *In trisomy 21 similar changes may* also be observed e g micropolygyria (1, 4) In other *autosomal syndromes, associated* with mental retardation, a diversity of cerebral malformations are described, some of *which resemble those of our case only in* the respect that

some of the malformations are based on a disturbance of migration

In sex chromosome abnormalities neuropathological studies are scarce *Forssman* (10) inclines to the view that the mental deviations seen in patients with sex chromosome aberrations are related to cerebral dysfunction, the basis of which may be an abnormality of structural neurochemical or neurophysiological type The possibility that intracranial vessels may be the site of abnormalities in patients with sex chromosome aberrations has also been suggested (18)

Concerning patients with the XYY syndrome we are not aware of any publication on the neuropathology As regards other types of sex chromosome abberrations we know only one case of a slightly mentally retarded girl with XO Turners syndrome (5), who had cerebral malformations of principally the same location and character as that in the present case The main points of difference relate to the smaller size of the brain less or no immature cellular remnants and less extensive heterotopias in the Turner case

Grossly the most prominent abnormality of the brain of the present case was its size With a weight of over 1700 grammes and in the absence of oedema and signs of storage disease it must be regarded as megalencephalic Direct relationship between brain size and body size is suggested by some authors though disputed by others Under no circumstances would the body size in the present case justify the recorded high weight of the brain In megalencephaly in its pure form developmental abnormalities similar to those in the present case e g heterotopias or cortical dysplasia have been reported (16)

Thus an XO or XYY chromosome constitution may not influence the basic pattern of the brain malformation but possibly the size of the brain

In the present case the gross impression of a gyral abnormality gained support from the histopathological findings of structural abnormalities in these areas These changes constitute a mild cortical dysplasia The diffusely scattered neurons in the white matter were too numerous and too deepseated to be normal and are diagnosed as diffuse heterotopias The small dark cells with sparse or no identifiable cytoplasm occurring in the molecular layer most likely represent undifferentiated embryonal cells The same interpretation goes for most of the similar cells in the hypothalamus and its basal meninges In these areas such remnants are sometimes found in association with malformations of the brain In the frontal hypothalamus such cuffs and meningeal infiltrates normally appear after the 4th intrauterine month and persist partly beyond birth (3) They are most likely remnants of a normal foetal developmental feature abnormally persisting postnatally Some of these cell infiltrates may be taken to indicate an encephalitic reaction though concomittant tissue destruction was entirely lacking making this interpretation less likely They appear to be a feature of retarded or arrested maturation

The changes in the cortical and white matter in our patient are thus interpreted as a malformation based on retarded migration and maturation

Disregarding megalencephaly the degree of mental retardation in the present case appears to be commensurate with the type degree and extent of malformations of the brain according to our experience from a large neuropathological material of mentally defectives Besides mental retardation such patients also often show psychiatric symptoms, e g passitivity, poor endurance emotional lability similar to those of the present case

Megalencephaly has not been reported in syndromes caused by chromosomal abnormalities though a familiar accumulation of cases with megalencephaly might arouse suspicion of a genetic background for at least some cases

In the experience of one of the authors (AB) the types of brain changes described in the present case are sometimes seen in mentally defective subjects without megal

encephaly and with no known chromosomal aberration In such cases, however, a certain familial tendency to similar cerebral malformations may again suggest a genetic background The pathogenetical role of the chromosomal aberration in the production of the cerebral abnormalities in our case is thus *difficult to evaluate A safe conclusion requires* careful studies of brains in other XYY cases and also of the chromosomal constitution in cases of mental retardation with similar types of cerebral malformations

Daly (7) reviewed 12 cases of the XYY syndrome from a neurological point of view and found as most consistent symptom an intention tremor and also noted impaired fine finger movements and body asymmetry The present case did not have intention tremor or body asymmetry but showed clumsiness of both fine and coarse motor movements From a neuropathological point of view there were *no changes that would have been expected to* cause intention tremor or go along with asymmetry of the brain development The conduction velocity of peripheral motor nerves in children with mental retardation is low, possibly pointing to delayed neuronal maturation This was thought to be related to the clumsy finger movements, particularly of the thumb seen in mentally retarded children (11) The nerve conduction velocity, however was not studied in the present case On the other hand, the mild cortical malformation may very well be responsible for his motor disturbance The lack of focal *neurological symptoms in the present case* agrees well with the lack of focal encephaloclastic changes

EEG abnormalities have been reported in some patients with the XYY syndrome, but *most XYY cases have had normal EEG s* (21, 31) More EEG studies in patients with the XYY syndrome are needed before any conclusion can be drawn A normal EEG as in the present case evidently does not exclude morphological cerebral abnormalities of the kind reported here

Our patient displayed a picture of pubertal testicular maturation with moderate degenerative processes The testes were *remarkable with regard to the early age of mature sperm* production The areal variation in maturation found in his testes is a common finding in normal pubertal testes The histology of the testes was quite different from that of 47, XXY Klinefelter's syndrome, *as in the latter the testicular tubules are mostly com*pletely hyalinized and Leydig cells are present in clumps The testicular changes in XYY men described in the literature (15 20) are similar to those of our patient Testicular, e g regressive, changes as in this case are, however, not uncommon in institutionalized mentally retarded patients (2)

This study was supported by grants from the *Swedish Medical Research Council* (12X 2037) and the *Prenatal Research Fund of the Stockholm newspaper 'Expressen'*

We thank Professor *Tom Saldéen*, Department of Forensic Medicine University of Uppsala Sweden for placing at our disposal the material necessary for this publication

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Acta path microbiol scand Section A *80*, 634–640, 1972

PLASMA CELL GRANULOMA (HISTIOCYTOMA) OF THE LUNG AND PLEURA

Report on Three Cases

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Some rare benign tumours may occur in the lungs and pleura, they are typically composed of plasma cells, lymphocytes, histiocytes, macrophages and various amounts of fibrous tissue These tumours are called histiocytomas (*Dubilier* 1968, *Ivanik* & *Mlekodaj* 1968, *Kinare et al* 1965, *Titus et al* 1962, *Wentworth et al* 1968), plasma cell tumours or plasmacytomas (*Childress et al* 1950, *Hellwig* 1934, *Legrand* & *Serfati* 1968, *Pätiälä* & *Perret* 1962), plasma cell granulomas (*Lane et al* 1955), xanthomas (*Scott et al* 1948), xanthomatotic pseudotumours (*Wentworth et al* 1968) Names such as "postinflammatory tumours" (*Umiker* & *Iverson* 1954), "sclerosing haemangioma" (*Liebow* & *Hubbe* 1956, *Rubin et al* 1958, *Turunen et al* 1957) and "vascular endothelioma" (*Edwards* & *Taylor* 1937) have also been used Because of this variety of nomenclature it is difficult to find out how many of the growths published under different names actually are granuloma-like histiocytomas of the lung However, the number of reported cases may be about thirty Similar pseudotumours in the pleura have also been reported (*Brown* & *Johnson* 1951, *Matsuk* 1956) In the present work, the authors present three cases of histiocytoma like tumours, two of which occurred in the lungs and one in the pleura The authors draw attention to the morphological similarity between the pleural tumours and the lung tumours

CASE REPORTS

Case 1 A 15-year-old schoolboy had been gaining weight abnormally since the age of 10 Otherwise he seemed to be healthy At 15, he weighed 97 kg and was 166 cm tall By routine chest x ray, a round opacity, 4 cm in diameter, was found in the right lung (Fig 1) No earlier chest x rays were available and thus, the time during which the tumour had developed could not be traced Before the x ray was taken, the patient had suffered from coryza for 9 months, but he had not been to a doctor At admission, the obese patient appeared healthy Heart sounds were normal without any murmurs and there were no rales in the lungs BP 130/70 Hgmm, P 84/min E S R 22, Hb 12 4 g/100 ml, W B C 8600/cmm normal differential count SGOT 11 IU/l, LDH 195 IU/l, urine analysis within normal limits The serum cholesterol was 316 mg/100 ml, triglycerides 4 20 IU/l, blood sugar 80 mg/100 ml, serum *electrophoresis* normal Bence Jones protein in the urine negative Microscopic examination of the bone marrow revealed a slight plasma cell reaction X-ray of the skull, long bones thoracic and lumbar vertebrae and pelvis did not show anything remarkable The excretion of 17-ketosteroids and 17-hydro-corticoids

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Fig 1 Routine chest x ray in Case I, showing a round opacity in the patient s right lung



Fig 2 Macroscopic picture of the tumour and the resected right middle lobe (Case 1)

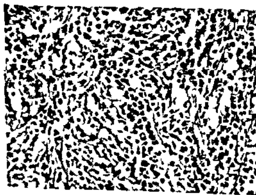


Fig 3 Microscopic appearance of the tumour in Case 1 showing mostly plasma cells and lymphocytes in dense groups and cords × 250 (H and E)

in the urine were within the normal limits Chromosome examination of the peripheral white cell culture revealed a normal XY caryotype

Bronchoscopy and examination of sputum did not reveal the diagnosis so thoracotomy was decided upon The lung was free from adhesions A round, dense tumour, 4 cm in diameter, was found in the right middle lobe which was resected The tumour was of hard consistency, it was well circumscribed but attached to the surrounding tissue and could not be bluntly dissected from it The cut surface of the tumour was whitish gray (Fig 2)

Microscopic examination showed a well circumscribed tumour, but no capsule was visible The growth extended under the visceral pleura, but was not connected to it The fibrous stroma was abundant The tumour was composed mainly of plasma cells and lymphocytes arranged in dense cell groups and cords (Fig 3) Some Russel bodies and small foci of calcification were observed There was no iron containing pigment nor were there any visible mast cells The general picture of the tumour was regular and a mitotic activity was not observed The pathological anatomical diagnosis was benign histiocytoma of the lung (plasma cell granuloma)

Recovery of the patient was uneventful Re examination 8 months later did not show evidence of recurrence The blood tests listed above were repeated but did not reveal anything remarkable The patient had been on a diet low in fats and carbohydrates His weight had decreased to 90 kg and the serum cholesterol (233 mg/100 mg) and triglycerides (3 66 IU/1) were normal

Case 2 A 20 year old female shop assistant whose grandmother had had a tuberculous spondylitis The patient had lived with her and was therefore examined several times including x ray of the chest In the summer of 1970 she had a cold and a temperature for some days In september 1970, routine chest x ray desclosed a coin shaped lesion in the right lung (Fig 4) which had not been visible at the routine chest x ray 18 months earlier

The patient was admitted to the local hospital for further exam[illegible]

[illegible]

Bence Jones protein in the urine negative Electrophoresis of the serum proteins revealed a normal distribution of the various fractions Examinations of the sputum for tuberculosis and tumour cells were negative Antituberculosis theraphy had no effect on the opacity The patient was admitted to surgery and, in January 1971, right thoracotomy was performed The lung was free from adhesions In the anterior segment of the right upper lobe a dense tumour, 3 cm in diameter was palpated, it

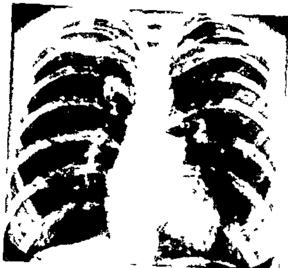


Fig 4 Routine chest x-ray in Case 2, showing the tumour in the right upper lobe

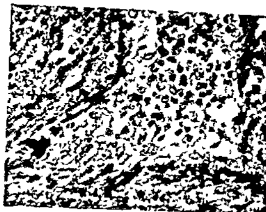


Fig 7 Histological section showing large, foamy cells intermingled with lymphocytes, plasma cells and mast cells in Case 2 × 250 (H and E)



Fig 5 Microscopic appearance of the well limited but not encapsulated tumour in Case 2 × 80 (H and E)

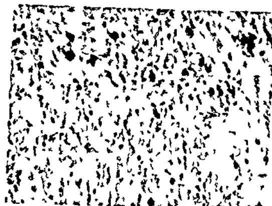


Fig 8 The mast cells of the tumour in Case 2 arranged into small groups × 250 (Toluidine blue stain)



Fig 6 Extension of the tumour beneath bronchial epithelium in Case II × 80 (H and E)

could not be enucleated from the lung tissue because of its attachments to the bronchus Consequently the whole upper lobe was resected

By microscopic examination the growth was found to be well defined but not encapsulated (Fig 5) Some groups of tumour cells were observed immediately under the bronchial epithelium which was intact (Fig 6) The cells of the tumour were partly elongated, and the fibrotic stroma was relatively sparse Most of the cells were histiocytes with abundant cytoplasm the latter giving a weak positive PAS reaction The histiocytes were arranged in groups Large, foamy cells were visible intermingled with lymphocytes plasmacells and mast cells (Fig 7) Variations in nuclear shape and size were only slight Rather low mitotic activity was observed No ironcontaining pigment was visible The mast cells were examined on toluidine-blue stained sections The mast cells arranged into small groups were numerous (Fig 8)

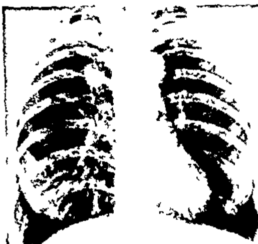


Fig 9 Chest x ray in Case 3, showing the pleural tumour in the upper field of the right lung

The pathological anatomical diagnosis was benign histiocytoma of the lung

Recovery of the patient was uneventfull and she was in good health at the follow up 6 months later

Case 3 A 44 year old female post office clerk, who had an epidemic parotitis at the age of 27 Since the age of 39 the patient had experienced several episodes of salpingoophoritis During the last two years, the patient had complained of a persistent cough Because of recurrent salpingo-ophoritis, the patient was admitted to the gynaecological out patient department in October, 1970 The patient was well developed, well nourished and in good general health Her temperature was slightly elevated (37 2 OC) The left ovarium was slightly enlarged and tender on palpation In the right cornu uteri, a small myomatous nodule was palpated The patient had a whitish discharge from the vagina Otherwise the physical examination did not reveal anything remarkable The patient received tetracycline theraphy and the gynaecological symptoms subsided

At routine chest x ray two weeks later a coin shaped lesion was noted in the upper field of the right lung (Fig 9) This opacity had developed after the routine chest x-ray had been taken 3 years earlier Because of this opacity, the patient was admitted for thoracic surgery The laboratory tests did not show anything remarkable E S R 30, Hb 12 7 g/100 ml, W B C 5000/cmm, normal *differential count SGOT 33 IU/1, LDH 140* IU/1, V D R T negative, electrophoresis of the serum proteins normal, urine analysis within normal limits, Bence-Jones protein in the urine negative At thoracotomy, a round, dense, well circumscribed tumour, 2 cm in diameter, was noted under the parietal pleura in the first intercostal space, 6–7 cm lateral to the sternum The tumour was extirpated It was of a hard consistency and the cut surface was greyish

Microscopic examination disclosed that the firm



Fig 10 Histological section showing the lymph node like architecture of the tumour in Case 3 × 80 (H and E)

growth was not encapsulated On one border of the tissue, an almost normal lymph node like architecture was observed (Fig 10) The tumour was composed of fibrotic stroma and inflammatory cells These cells were mostly lymphocytes and plasma cells but some neutrophil granulocytes were also visible In toluidine stain a moderate number of mast cells was observed No eosinophil granulocytes or foamy cells were observed There were some single mitoses but generally the mitotic activity was rather low Staining for iron gave a negative result Pathological anatomical diagnosis plasmacell granuloma of the pleura

Recovery of the patient was uneventful At follow up 8 months after operation, the patient said that the cough which prior to operation had been persistent by now had subsided The laboratory tests and chest x ray did not show anything remarkable

DISCUSSION

Whenever the diagnosis histiocytoma of the lung is to be established, it seems important to have it distinguished from plasmocytoma In the cases first reported in the literature this differentiation had not been performed and both tumours had been recorded as if they potentially were equally malignant (*Cotton & Penido* 1950, *Hill & White* 1953) It can hardly be doubled that cases of genuine plasmocytomas may have been comprised in these series and thus recurrences are easily explained (*Childress & Adie* 1950, 1955) Sometimes the lung manifestations of malignant plasmocytoma may cause the first complaints and it is especially important in these cases to have them distinguished from plasma cell granulomas (*Rozsa & Frieman* 1953, *Rimbaldi et al* 1959, *Robson & Knudsen* 1959 *Legrand & Serfati* 1968, *Viszloy & Daroczy* 1960, *Romanoff & Milavidsky* 1962) The plasmocytomas are composed exclusively of plasmacells, in the malignant cases, atypical nuclei and mitoses are frequent In the histiocytomas atypical cells do not occur and mitoses are usually only few

In cases of pleural tumours, differentiation between mesotheliomas and histiocytomas has to be carried out Mesotheliomas without fibrosis, are characterized by a presence of mesothelial cells resembling epithelial cells, the latter may even form papillary structures Lymphocytic, plasmacell, histiocytic and macrophage reactions are not typical of mesotheliomas, they occur frequently also in histiocytomas (*Foster & Ackerman* 1960)

Although the different authors agree about the occurrence of plasmocytic and histiocytic benign tumours of the pleura and lungs, the nomenclature is still unsettled Spencer (1962) used the subgroups histiocytoma, plasmacell granuloma and sclerosing haemangioma (vascular endothelioma) According to Titus, these tumours are histopathologically very similar the only difference being a difference in predominating element According to the predominating element these tumours can be called 'histiocytoma 'plasmacell granuloma', sclerosing haemangioma" or inflammatory pseudotumour (*Titus et al* 1962) Because of the unknown aethiology and development of these tumours evidence has not yet been produced by which Titus theory might be questemed However these subgroups have no importance in the treatment of the patient

Based on the clinical and radiological features, the diagnosis of histiocytoma can be suspected only when there is a round opacity in the lungs The diagnosis can be confirmed by histological examination on the surgical specimen Generally the tumours have no communication with the larger bronchi, although Bates & Hull in 1958 reported one case of this type

The aetiology of the histiocytoma is unknown Certain signs indicate that it could be an inflammatory process. All patients in the present series had a history of infection some months before the diagnosis of histiocytoma was established A previous respiratory infection had also been manifest in some of the cases reported in the literature (*Umiker & Iverson* 1954, *Cotton & Penido* 1952 *Bates & Hull* 1958, *Dubilier et al* 1968 *Fischer & Bayer* 1959) Similar tumours have occurred in man after accidental contamination with the Yaba virus (*Grace et al* 1962) and experimentally it is possible to produce benign histiocytomas in monkey with Yaba

viruses (*Sproul et al* 1963) Although the cells of these growths are typical of chronic inflammation, the tumour like structure and the intensity of the cell reaction is atypical It is therefore uncertain whether these tumours should be considered inflammatory or neoplastic in the lungs as well as in other organs

All of the patients presented in this paper were young or middle aged, which is in conformly with descriptions in the literature Although these tumours usually occur in young adults, they have been reported to occur in almost all age groups under 70 Because the tumours cause little complaints, they are difficult to diagnose and thus their age is difficult to determine The complaints caused by these tumours are not specific. They may be coughs respiratory infections or, as reported in one case by Hill in 1953, even clubbing of the toes and fingers Most of the reported cases were found accidentally at routine chest x-ray examination Some of these tumours may remain undetected because of the lack of specific symptoms Only one out of our three patients had a complaint of 2 months' duration In case no 2, no tumour had been noted 18 months previously, so we could perhaps say that this tumour developed relatively quickly In the other two cases, certain complaints had been manifest for a couple of months A chest x ray of one of these patients, taken three years previously was available but no signs of a tumour were visible

The histiocytomas are usually well circumscribed but not encapsulated Macroscopically a yellow colour will often be observed the tumour may be a site haemorrhages (*Dubilier* 1968) The tumours are of different size the largest on record measuring about 10 cm in diameter (*Ivanik & Mlekodaj* 1968)

In the opinion of the various authors, histiocytoma is a benign tumour which does not recur after total extirpation Because excision of the tumour is the adequate therapy, the possibility of histiocytoma of the lungs has to be taken into consideration even before operation and the actual nature of the tumour has to be determined by the frozen section technique during the operation

One of the tumours reported in the present paper was localized to the pleura Although cases of pleural tumours have been reported in the literature, comments on the similarities between benign plasmocytic and histocytic tumours of the lung and pleura are few only

Histiocytoma of the lungs and pleura is a rare benign tumour occurring mostly in young patients The numerous diagnoses used to describe these tumours have caused some differential diagnostic difficulties which are discussed Three cases seen by the authors are presented, they were all detected by radiological examination of the chest Attention is paid to the similarity between histological features in the lungs and in pleural location, and to the possible infectious aetiology of these tumours

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Acta path microbiol scand Section A 80, 641-649 1972

INFLUENCE OF AGE AND SEX AND OF CASTRATION ON RENIN SUBSTRATE OF NORMAL AND NEPHRECTOMIZED RATS

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The average renin substrate concentration in 15 day-old female and male Wistar rats was about 300 expressed as ng angiotensin per ml With increasing age it rose rapidly to a maximum of about 400 ng in females, and more slowly to about 700 ng in males Castration of mature or immature rats of both sexes did not influence either renin substrate or the degree of its increase following nephrectomy The average postbinephrectomy values was about 3100 ± 1000 ng in both sexes, being about 9 times increased in mature females and about 6 times in mature males In immature females the postbinephrectomy values had increased by about 8 times up to about 2400 ng Renin substrate seems to be markedly influenced by changes in the environment

The influence of age on plasma renin substrate concentration in rats has apparently not been studied previously, and studies of the influence of sex have given contradictory results (*Carretero & Gross* 1967, and *Nasjletti et al* 1971) *Helmer & Griffith* (1952) and many subsequent investigators (for literature see *Page & McCubbin* 1968) found increased values after treatment with oestrogens and *Nasjletti et al* (1971) found a significant increase during oestrus They also showed that renin substrate was reduced 5 days after castration in oestrus rats, but not in dioestrus rats and that it was unchanged 5 days after castration of male rats

The present paper forms part of studies of the mechanism of postbinephrectomy increase in renin substrate It aims at an elucidation of the influence of age and sex, including both normal and nephrectomized previously sham operated or castrated rats, these operations were performed either on mature animals or on young rats weighing about 25-30 g and the renin substrate was determined after an interval of a few days up to well over one month

MATERIAL AND METHODS

Female and male specific pathogen free Wistar rats of strain Af/Han/Mo (Han 67) were used As regards the young rats (30 to 70 g) blood was obtained during light ether anaesthesia after decapitation and in the case of older rats it was drawn from the carotic artery In the case of rats weighing 30 to 37 g blood from two animals was pooled The blood was at once cooled to 4°C 50 µl of a 6 per cent sodium citrate solution being added per ml blood In order to make the comparison as independent of environmental influences

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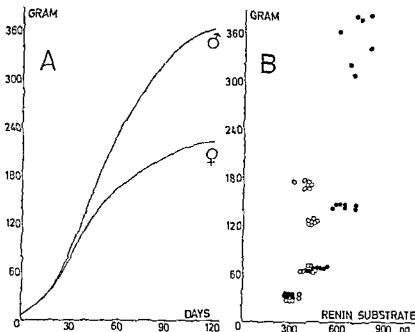


Fig 1A Shows the relation between body weight in g and age in days in female and male rats of the strain of Wistar rats used in the present study and *Fig 1B* the relation between body weight and renin substrate in ng angiotensin per ml plasma The values from female rats are marked O, and those of males ●

as possible the same number of sham operated and castrated rats were operated and later bled on the same days.

Renin substrate was determined by radio immuno assay for angiotensin I using a slight modification of the method of *Poulsen* (1969)

RESULTS

1 *Effect of Age and Sex*

The *body weight* of rats belonging to the strain used varies with age as shown by the two lines in Fig 1A, which represent the mean values from studies of 150 females and 150 males and show the higher increase with age in male than in female rats Fig 1B shows that identical *renin substrate* values of about 300 ng are found in females and males weighing about 30 g, but that the values of the two sexes differ in older rats In females a maximum of about 400 ng is reached and maintained when they weigh more than 60 g while there is a more prolonged rise in the males in which values of about 700 ng are found when they weigh about 350 g

2 *Effect of Castration*

That castration of *female* rats did not result in any changes in renin substrate is seen in the left half of the upper row of columns in Fig 2 which shows the mean values ± SE in females sham operated (hatched column) or castrated, whether mature or immature (body weight about 30 g) The mature animals were bled 3 to 34 days after operation the immature ones 2 to 4 months after the operations, these differences in time being without effect on the results In the mature rats the mean values were 331 ng ± 76 in the sham operated and 374 ± 79 in the castrated, the difference being non significant both in these ($p > 0.8$) and in the smaller number of rats operated during immaturity ($0.2 > p > 0.1$)

A similar lack of effect of castration on renin substrate was found in *male* rats (right half of upper row in Fig 2) The mean values of those operated during maturity (weight about 300 g) was 561 ± 127 in the sham operated and 475 ± 120 ng in

the castrated, (0 1 > p > 0 05), very similar values being found in the males which were castrated when they were about 15 days old and bled when they weighed about 260 g (0 4 > p > 0 3)

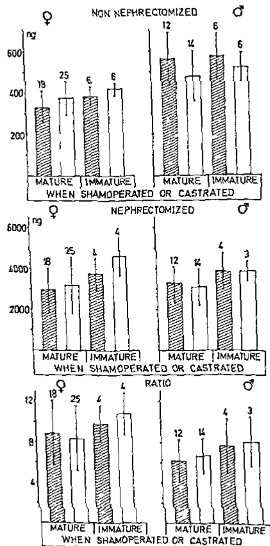


Fig 2 Shows the mean values ± SE for renin substrate in ng angiotensin per ml of plasma in sham operated (hatched columns) and castrated rats (non hatched columns) The *upper* row shows the values before binephrectomy the *middle* row those 24 hours after nephrectomy and the *bottom* row the ratio indicating how may times renin substrate increased following binephrectomy The number at the top of a column indicates the number of rats in this particular group

41*

3 *Effect of Binephrectomy*

The 24 hours' post binephrectomy increase in renin substrate was studied in the above mentioned rats Fig 2 (middle row) shows again that there were no significant differences between rats sham operated and castrated during maturity, neither in females nor in males (p > 0 5) While renin substrate was significantly higher in normal males than in normal females, the post binephrectomy values of the two sexes do not differ significantly (0 7 > p > 0 6), the mean values of both being about 3100 ng ± 1000 in the animals operated during maturity The *ratio* between the pre and post nephrectomy values (Fig 2 bottom row), indicating how many times the substrate concentration was increased 24 hours after the binephrectomy, is therefore significantly (0 005 > p) higher in female (8 6 ± 3 1) than in male rats (6 5 ± 1 9)

In 12, previously unoperated, 15 day old rats, binephrectomy was followed by an approximate 8 times increase in renin substrate which rose from about 300 ng to about 2400 ng

4 *Effects of Environmental Influences*

When renin substrate of rats of the same sex and age was determined at long intervals of time the concentration would often remain about the same, but sometimes the values might differ markedly Examples of this are given in Fig 3 Values in the mature female rats in the first two groups which are identical with the group of mature females shown in Fig 1 and the sham operated females in Fig 2 respectively differ markedly (0 005 > p) from the values obtained in a group studied much later The difference (0 02 > p > 0 01) between the two groups of males depicted in Fig 1 and 2, respectively is also found to be significant Similarly, values (0 005 > p) found in the second group of immature female rats weighing about 60 g are markedly higher than those in the two other groups which do not differ significantly

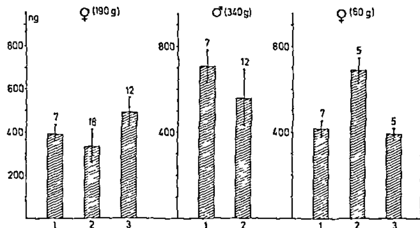


Fig 3 Shows that although differences in renin substrate concentration are only insignificant in rats of identical age and sex kept under uniform conditions (as the mature and immature animals, respectively in Fig 2), differences may be marked in rats studied at different times with consequent possibility of changes in their environment Examples of this based on studies of mature rats of both sexes and of immature female rats are given the columns showing mean values ± SE of renin substrate in ng angiotensin per ml of plasma in different groups the number of animals in each group is stated above the column

DISCUSSION

The present study has shown that renin substrate of rats is identical in *the two sexes* while they are quite young, but increases in different ways with growing age and maturity, reaching about 1 5 times as high values in males than in females This is about the same ratio as that found by *Carretero & Gross* (1967), but the values of 350–450 ng in females and 600–800 ng in males are markedly higher than values found by these authors

These differences between the renin substrate concentrations of the two sexes and the above mentioned influence of oestrogens suggested that *castration* would influence renin substrate concentration This was not found, however, whether the animals were castrated while immature, or castration was performed on mature rats which agrees with the findings by *Nasjletti et al* (1971) using dioestrus female and male rats

That the *postbinephrectomy increase in substrate* is independent of the gonads appears from the fact that values were identical in sham operated and castrated rats These further studies show that, after binephrectomy, the renin substrate is *relatively* more increased in females than in males with the result that the postbinephrectomy values in the two sexes are about the same about 3100 ± 1000 That this does not mean that the substrate concentraion cannot reach higher values, is known from previous studies, showing still higher values in simultaneously nephrectomized and oestradiol treated rats (*Nasjletti et al* 1969) and in simultaneously nephrectomized (or ureterligated) and hypoxic rats (*Bing & Poulsen* 1969)

The finding that the renin substrate values in groups of rats of the same strain age and sex are not always constant can be explained either by undiagnosed abnormalities in the rats or by environmental influences (A third possibility changes due to the technique used for the analyses, were eliminated by constant use of a standard plasma) In the examples given in Fig 3 the abnormally high values of the second column of the immature females weighing about 60 g were probably due to insufficient regulation of the temperature and air condition in the animal room the values returning to normal in the third group studied after the regulation of temperature and air condition had been restored Such influence of the environment raises the

question whether renin substrate may be related to the acute phase proteins which are easily increased by various exogenous and endogenous influences

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Supported by grants from *King Christian X's Foundation* We are further grateful to vet Dr *Møllegård*, who kindly placed the values given in Fig 1A at our disposal

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RELATION BETWEEN RENIN SUBSTRATE AND ACUTE PHASE PROTEINS

Studies of Normal and Adrenalectomized Rats

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In adrenalectomized rats, renin substrate was as previously found extremely low in non substituted animals, markedly decreased in salt substituted animals, but only slightly de creased in both salt and DOCA substituted animals After three of four injuries known to increase acute phase proteins, there was also an adrenal dependent increase in renin sub- strate A similar adrenal dependent increase in substrate was further found in 24 hour uninephrectomized rats and in rats with ceased urine excretion, while the increase after binephrectomy is non adrenal dependent and thus not due to ceased urine excretion

The concentration of renin substrate is in fluenced by many factors (see *Page* & *Mc Cubbin* 1968, and *Lee* 1969), of which adre- nal cortical steroids and, therefore also ACTH cause an increase The increase found after different operations (*Romero et al* 1970 and *Blaine et al* 1971), is adrenal de pendent (*Lazar* & *Hoobler* 1971)

The present study was caused by two find ings 1) that ceased urine excretion, caused by vesico venous anastomosis is followed by higher increase in substrate in rats (*Bing* & *Jorgensen* 1972a) than in dogs (*Blaine et al* 1971), and 2) that changes in the environ ment influence the substrate concentration (*Bing* & *Jorgensen* 1972c) These findings raised the question whether renin substrate is related to the acute phase proteins which are characterized by their increase after dif ferent forms of injury such as those found after injection of celite, turpentine or bac terial pyrogens and after partial hepatectomy (*Heim* & *Ellenson* 1965, *Gordon* 1970, *Sar cione* 1970, *Lynn* & *Sidransky* 1970) The aim of this paper was to show whether such injuries also cause an increase in renin sub strate, and if so, whether the increase is adre nal dependent in the same way as that of most acute phase proteins (*Weimar et al* 1966 and 1967) As a basis for these studies the effect of adrenalectomy without and with substitution with DOCA and (or) salt was reinvestigated It was finally studied whether the increased substrate after uni nephrectomy and after ceased urine excre tion caused by intravenous re infusion of urine is adrenal dependent

MATERIAL AND METHODS

Animals female S P F Wistar rats weighing 180 220 g unless otherwise stated provided with a commercial chow and tap water *Adrenalectomy partial hepatectomy* (removal of about 55 per cent of the liver by subtotal removal of the left and the middle lobes) *uni and binephrectomy* were per- formed on penicillin pretreated ether anaesthetized rats *Salt substitution* 1 per cent sodium chloride

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as drinking fluid *DOCA* a daily dose of 0 15, 0 45 or 1 5 mg desoxicortone acetate given s.c in oil *Celite* a suspension of 11 mg of celite 545 (Johns Manville) in 5 ml sterile saline i p *Turpentine* 0 15 ml i m in each tigh and 0 5 ml s c. of a sterile 50 per cent solution in oil *Bacterial pyrogen* 0 2 ml i v of a suspension of 100 millions killed bacteria of the faecal s alcaligenes group per ml (State Serum Institute) Ceased

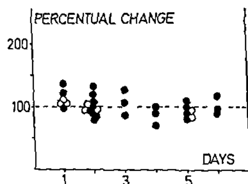


Fig 1 The constancy of renin substrate, independent of a small bleeding 1 5 ml in normal (●) and adrenalectomized rats substituted with 0 15 mg DOCA per day and (or) saline (O) Ordinate the renin substrate concentration of the second sample in per cent of that of the first Abscissa days between the two samplings

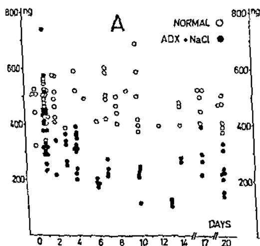


Fig 2 A Renin substrate concentration in ng
nalectomized (●) rats
B substrate in adrenalectomized rats which wer
0 15 to 1 5 mg DOCA per day (+), the differ
cissa in A and B days after adrenalectomy
C shows the ratio, that is to say how many tin
after binephrectomy, showing that about the sa
in both salt and DOCA (+) substituted adren

ng (○ Fig 2A) for three weeks Contrary to this a continuous pronounced decrease was found in the *non substituted adrenalectomized* animals (△ Fig 2B), the values after two weeks being only 25 50 ng In the *salt substituted adrenalectomized* (● Fig 2A) the values fell in about five days to 200–300 ng while *salt and DOCA substituted* rats (+ Fig 2B) had values of 300–400 ng during the first two weeks and about 300 ng after three weeks independent of variations in the dosage of DOCA from 0 15 to 1 5 mg per day

The *24 hour post binephrectomy increase in renin substrate* was studied in some of the rats The ratio indicating how many times the substrate concentration increased following binephrectomy is given in Fig 2C which shows that the *relative* increase is about the same in normal rats (○) and in salt (●) or salt and DOCA (+) substituted adrenalectomized rats Simultaneously studied non substituted adrenalectomized rats died less than 24 hours after binephrectomy

2 *Changes in Renin Substrate of Normal and Adrenalectomized Rats after Injuries which cause Increase in Acute Phase Proteins*

Three out of four forms of injury which are known to be followed by increase in the plasma of the acute phase proteins namely 1) intraperitoneal injection of celite 2) partial hepatectomy and 3) intravenous injection of bacterial pyrogens produced more or less marked increase in renin substrate while the fourth 4) intramuscular and subcutaneous injection of turpentine was without effect (● Fig 3) Because of the adrenal dependency of most acute phase proteins the four types of injury were also performed on adrenalectomized rats substituted with 0 15 mg DOCA and (or) sodium chloride (○ Fig 3) The figure shows that also the effect in renin substrate is adrenal dependent After partial hepatectomy and after pyrogen injections some adrenalectomized rats had very low substrate values the plasma of these animals being icteric as a sign of liver damage Such low values were not found when the daily dose of DOCA was increased from 0 15 to 0 45 or 1 5 mg (△ Fig 3) in pyrogen treated rats

3 *Effect of Uninephrectomy and of Ceased Urine Excretion on Normal and on Adrenalectomized Rats*

It applies to the 24 hour uninephrectomized rats as well as to most rats which for 6 hours had ceased urine excretion because of reinfusion of their own urine that there was an adrenal dependent increase in renin substrate (Fig 3) Some of the adrenalectomized urine re infused rats had values as low as those found in some of the partially hepatectomized or pyrogen injured rats

DISCUSSION

1 *Effect of non Substituted or Substituted Adrenalectomy on Renin Substrate of Normal and Nephrectomized Rats*

The reinvestigation of the effect of adrenalectomy was performed because of differences in the results of previous investigations. The present study shows a marked fall in substrate concentration to less than 10 per cent of the normal in *non substituted* rats (△ Fig 2B) a less pronounced progressive fall in *saline substituted* (● Fig 2A) and about 25 per cent decreased values in both *saline and DOCA substituted* animals (+ Fig 2B) The non adrenalectomized controls (○ Fig 2A) had a substrate concentration about 25 per cent higher than that previously found in this strain (*Bing & Jorgensen 1972c*) which can explain the decrease in the saline and DOCA substituted rats if the unknown probably environmental cause of the increase is adrenal dependent in the same way as the increases found after the injuries shown in Fig 3

The finding of a fall to extremely low substrate concentrations in *non substituted* animals is in accordance with all (*Lewis & Goldblatt* (1942)

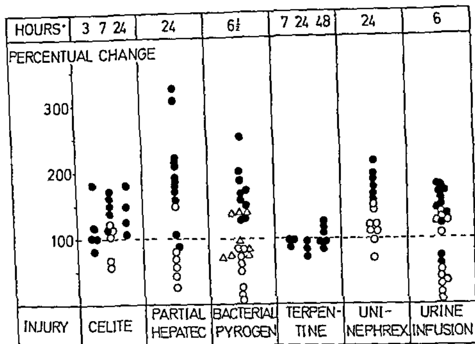


Fig 3 The percentual change in renin substrate concentration (given in the same way as in Fig 1) at different times (given in hours above) after different types of injury (given below) in normal (●) and in adrenalectomized rats, substituted with salt and DOCA in a dosage of 0 15 mg (○) or 0 45 to 1 5 mg (△) per day

Houssay & Dexter (1942) *Helmer & Griffith* (1951) *Carretero & Gross* (1967) *Nasjletti et al* (1969) *Tateishi et al* (1971) *Dauda & Devenyi* (1971) but one (*Lazar & Hoobler* (1971)) of the previous investigations The progressive fall in *saline substituted* animals was also found by *Helmer & Griffith* (1951) and *Haynes et al* (1952) but not by *Nasjletti et al* (1969), *Lazar & Hoobler* (1971) and *Dauda & Devenyi* (1971) The slight fall in both *saline and DOCA substituted* animals the probable cause of which is given above has only been found by *Helmer & Griffith* (1951) but neither by *Haynes et al* (1952) *Nasjletti et al* (1969) nor by *Lazar & Hoobler* (1971)

The finding that *binephrectomy* results in about the same *relative* increase in renin substrate in normal and adrenalectomized rats (Fig 2C) is in agreement with all (*Gaudino* (1944), *Carretero & Gross* (1966) and *Tateishi et al (1971)) but one (Lazar & Hoobler* (1971)) of the previous investigations The effect of binephrectomy is thus a stimulation of the production and liberation of renin substrate in the liver (*Nasjletti & Masson* (1971) and *Tateishi & Masson* (1972)), resulting in a multiplication of the plasma concentration independent of both its initial level and of adrenal function

2 *Relation between Renin Substrate and Acute Phase Proteins*

The present finding of an adrenal dependent increase in renin substrate after injuries which increase the acute phase proteins indicates a relation between these otherwise quite different proteins, probably due to their common hepatic origin The finding of a similar adrenal-dependent increase in renin substrate after uninephrectomy and after ceased urine excretion shows that these two types of injury are related to those which induce the increase in acute phase proteins Such unspecific reaction to injury is especially pronounced in rats and explains why ceased urine excretion resulted in a more pronounced increase in renin substrate in rats than in

dogs The conclusion of *Blaine et al* (1971), that the increase in substrate after experimental uraemia is probably due to stress induced ACTH release is further supported by the adrenal dependency shown in Fig 3 The pronounced postbinephrectomy increase in substrate is, contrary to that found in experimental uraemia, *not* adrenal dependent, and accordingly, it cannot be explained by the ceased urine excretion, but must have another, still unknown cause

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Supported by grants from *King Christian X s foundation*

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FIBRINOLYTIC ACTIVITY OF RENAL TRANSPLANTS IN RABBITS RELATION TO GRAFT THROMBOSIS AND NECROSIS

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The fibrinolytic activity of renal transplants in rabbits was studied by the fibrin slide technique a histochemical substrate film method for the demonstration of tissue activator of plasminogen Biopsy and graftectomy specimens from seven autografts four allografts and eight allografts from recipients presensitized against the donor kidneys were studied The findings were semiquantitated and compared with the presence of graft thrombosis and necrosis All the grafts demonstrating a decrease in fibrinolytic activity after transplantation developed thrombosis and cortical necrosis and all the grafts showing thrombosis and necrosis had decreased fibrinolytic activity Only one case with focal cortical necrosis in the graft without any thrombi had no decrease in fibrinolytic activity of the graft The decrease in fibrinolytic activity seemed to precede the occurrence of thrombosis and necrosis

Since the first reports on the hyperacute rejection of human renal allografts (*Kissmeyer Nielsen et al* 1966 *Terasaki et al* 1967 *Williams et al* 1967 *Starzl et al* 1968), in which microthrombosis and necrosis is a hallmark the importance of a local intravascular coagulation in the pathogenesis of the early allograft rejection has been emphasized (*Lichtman et al* 1968, *Busch et al* 1969 *Colman et al* 1969 *Myburgh et al* 1969 *Rosenberg et al* 1969) The demonstration of urine fibrinogen split products during acute rejection reveals the implication of the fibrinolytic system too (*Braun & Merrill* 1968 *Carlsson et al* 1970) As a preliminary rough hypothesis of pathogenesis in organ rejection it is suggested that circulating antibodies directed against HL A antigens on plasma membranes of the graft initiate a complex sequence of biologic responses in which endothelial and smooth muscle cells of the blood vessels are injured probably through the action of serum complement and polymorphonuclear leucocytes Platelets aggregate on surfaces of the blood vessels denuded of endothelium and fibrin is precipitated (*Busch et al* 1971) By activation of the fibrinolytic enzymes considerable amounts of fibrin can be dissolved Concomittantly other enzyme systems are activated and highly vasoactive compounds may be formed If the capacity of fibrin clearing is overwhelmed obliterative thrombosis may ensue, and ischaemic necrosis develops

The fibrin clearing mechanism of the organism is mainly due to the proteinase, plasmin formed by activation of the circulating

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precursor, plasminogen. Activators of plasminogen are widely distributed in the tissues of mammals (*Astrup* 1966, 1970). By a histochemical substrate film method, the fibrin slide technique (*Todd* 1958, *Kwaan* & *Astrup* 1967), it has been shown that blood vessels generally are highly fibrinolytically active, endothelial cells being rich in plasminogen activator. Other tissues and cells, however, are fibrinolytically active as well (*Pandolfi* & *Astrup* 1967, *Henrichsen* & *Astrup* 1967, *Tympanidis et al.* 1968, *Myhre-Jensen et al.* 1969, *Myhre-Jensen* & *Astrup* 1971, *Myhre-Jensen et al.* 1971). In the kidney of healthy rats and rabbits, for instance, plasminogen activator is localized in the blood vessels, vasa rectae of the renal medulla being highly active, peritubular capillaries being only weakly and inconstantly active and other blood vessels including glomeruli being intermediary in activity. Weak fibrinolytic activity is related to papillary ducts and inconstantly to collecting ducts and tubules. Pelvic, ureteral and bladder mucosa is highly fibrinolytically active (*Myhre-Jensen* 1971).

The results of a study of the morphological alterations in kidney grafts in rabbits have recently been published (*Lund* & *Myhre-Jensen* 1970 a, b, 1971 a, b). Because of the implication of the process of fibrin deposition and clearing in the mechanism of early graft rejection, specimens from the kidney grafts were studied by the fibrin slide technique. Comparison of the fibrinolytic activity of the grafts with the occurrence of thrombosis and necrosis was done.

MATERIAL AND METHODS

Randomly bred adult rabbits weighing between 2 and 3 kg were used. Kidney transplantation was performed by end to side anastomosis between the renal vessels of the left donor kidney and the abdominal aorta and the inferior vena cava of the recipient. Warm ischaemia lasted a few minutes, cold ischaemia for about 40 minutes. One of the recipient's own kidneys remained untouched. No immunosuppressive therapy was given. The operative technique, postoperative care of the animals and complications are previously described (*Lund* 1970). Wedge biopsies were taken from the grafts. The number of biopsies in individual grafts and intervals after transplantation appear in *Table I*. In 7 autografts 27 biopsies (34 specimens in all), in 4 allografts 10 biopsies (14 specimens), and in 8 allografts from recipients presensitized by injections of donor kidney homogenate 16 biopsies (24 specimens) were performed. Furthermore, in two non transplanted kidneys, made totally ischaemic by clamping of the pedicle, 12 biopsies (14 specimens) were obtained.

Tissue specimens were immediately frozen at about —70° C and stored at —60 to —70° C in sealed vessels for a maximum of a few months before the study with the fibrin slide technique. Experience from previous studies has shown that tissue activator of plasminogen is preserved for several months under these circumstances. Frozen sections, 6–8 μ, were incubated with plasminogen rich fibrin in a moist chamber at 37° C, fixed in 4 per cent neutral formaldehyde solution and stained in Harris' haematoxylin. Control sections were incubated with plasminogen free fibrin in order to detect free, unspecific proteinase activity. Microscopically, sites with fibrinolytic activity appear as clear digested zones in the stained fibrin film. Fibrinolysis caused by an activator of plasminogen produces lysis only on slides prepared with the plasminogen rich fibrinogen. Nonspecific proteinases will produce lysis on plasminogen free fibrin as well.

From one specimen, several slides which contained two or three frozen sections were prepared. In one series of slides the fibrin films were formed on top of and covering the sections, in the other series from the same specimen the sections were picked up from the cryostat directly on the fibrin films covering the slides. In each series individual slides were incubated for 5, 10, 15, 20, 30, 40, 50, 60, 75, 90 and 120 minutes. In kidneys of the healthy rabbits the first indication of lysis appears after about 5 minutes incubation with this technique. Less active sites, however, produce lysis only after up to 60–75 minutes incubation. After incubation periods of that long duration, zones of lysis around highly active structures usually become large and confluent and after 90 or 120 min. incubation extend beyond entire section.

Based on the findings obtained by the fibrin slide technique and compared with normal rabbit kidneys the activity of transplanted and clamped kidneys were semiquantitated. | = fibrinolytic activity within the range of the normal kidney. ↓ = slight decrease in activity, i. e. few active sites in renal cortex within 120 min. incubation. ↓↓ = intermediary decrease in activity, i. e. no active sites in renal cortex and few active sites in renal medulla (only the outer zone was represented in the wedge biopsies) within 120 min. incubation. ↓↓↓ = marked decrease in activity, i. e. no fibrinolysis at all in cortex and medulla within 120 min. incubation.

TABLE 1 *Correlation of Renal Fibrinolytic Activity and Thrombosis and Necrosis Kidney Transplantation and Clamping in Rabbits*

	Rabbit	Interval after transplantation	Fibrinolytic activity	Thrombosis Glomeruli	Thrombosis Other vessels	Necrosis
Autografts	374	1 hour	I	0	0	0
		1 day		0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
		4 days	I	0	0	0
		5 days	I	0	0	0
	375	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
		4 days	I	0	0	0
		5 days	I	0	0	0
	376	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
		4 days	I	0	0	0
		5 days	I	0	0	0
	377	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
		4 days	I	0	0	0
		5 days	I	0	0	0
	379	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
	380	1 day	↓	0	0	0
		2 days	↓↓↓	0	+ + +	+ +
	381	2 days	I	0	0	0
		7 days	I	0	0	0
		14 days	I	0	0	0
		21 days	I	0	0	0
Allografts	443	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
	444	1 hour		0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
	445	1 hour	I	0	0	0
		1 day	I	0	0	0
		2 days	I	0	0	0
		3 days	I	0	0	0
		4 days	I	0	0	0
		5 days	I	0	0	0
	446	1 hour	I	0	0	0
		3 days	I	0	0	+

TABLE 1 *(Continued)*

	Rabbit	Interval after transplantation	Fibrinolytic activity	Thrombosis Glomeruli	Thrombosis Other vessels	Necrosis
Allografts, presensitized recipients	261	1 day	↓	0	0	+ + +
		2 days	↓↓↓	0	+ +	+ +
	263	1 day	|	0	0	0
		2 days	|	0	0	0
	271	1 day	|	0	0	0
		3 days	|	0	0	0
		4 days	|	0	0	0
	277	1 day	|	0	0	0
		4 days	|	+	+	0
		6 days	↓	+	+ +	0
	279	1 day	|	0	0	0
		3 days	|	0	0	0
	281	1 day	|	0	0	0
		3 days	↓↓↓	0	+ + +	+ + +
	285	1 hour	↓	0	0	0
		1 day	↓↓	+ +	+ + +	0
		2 days	↓↓↓	+ + +	+ + +	+ + +
		3 days	↓↓↓	+ + +	+ + +	+ + +
		4 days	↓↓↓	+ + +	+ + +	+ + + +
	291	2 hours	|	0	0	0
		1 day	↓	0	0	0
		2 days	↓	0	0	0
		3 days	↓	0	+	+
		4 days	|	0	+ +	+

	Rabbit	Interval after clamping	Fibrinolytic activity	Thrombosis Glomeruli	Thrombosis Other vessels	Necrosis
Non transplanted	601	2 hours	↓	0	0	0
		4 hours	|	0	0	0
		6 hours	↓	0	0	0
		8 hours	↓↓	0	0	0
		1 day		0	0	0
		2 days	↓↓	0	+	+ + +
	602	1 hour		0	0	
		2 hours	↓	0	0	0
		4 hours	↓	0	0	0
		6 hours	↓↓	0	0	0
		8 hours	↓	0	0	0
		1 day	↓↓	0	0	0
		2 days	↓↓↓	0	+ +	+ + +

Focal lysis time i e the shortest incubation time required to produce a clearly demarcated zone of lysis at an active area could not be used as a semi quantitative measure of fibrinolytic activity In specimens with a moderate decrease in activity the few active sites had focal lysis times within or slightly above the range of that of the normal rabbit kidney The presence of thrombosis and necrosis was studied in paraffin sections from formaldehyde fixed biopsy and graftectomy specimens stained with haematoxylin eosin periodic acid Shiff and phosphotungstic acid haematoxylin

RESULTS

The results are shown in *Table 1*. In seven *autografts*, one (380) had partial cortical necrosis and massive thrombosis of graft vessels. Fibrinolytic activity was definitely decreased in the graft removed 2 days after transplantation. The graft biopsy from the first day, by which time the necrosis and thrombosis was not evident, had only a moderate decrease in fibrinolytic activity. In four *allografts* focal cortical necrosis without thrombosis was present in one renal transplant (446). Fibrinolytic activity, however, was within the range of that in the normal rabbit kidney. In the group of *allotransplanted, presensitized* rabbits only three renal grafts among 8 exhibited no cortical necrosis or thrombosis. Fibrinolytic activity in those three grafts was normal. In specimens from five grafts with cortical necrosis and thrombosis (261, 277, 281, 291) fibrinolytic activity was decreased in all. In one case (285), showing a massive graft necrosis 4 days after renal transplantation and extensive thrombosis of blood vessels and glomeruli, fibrinolytic activity, which already in the biopsy one hour after transplantation was moderately decreased, became almost entirely extinct (Fig 1). In two *clamped, non transplanted* rabbit kidneys there was a general tendency to a decrease in fibrinolytic activity with the time after clamping, being definitely decreased in one kidney (602) at the time of removal two days after clamping. Renal cortical necrosis and a moderate thrombosis, however, became evident only in the second day specimen. Thus, in that experiment, the decrease in fibrinolytic activity seemed also to precede development of renal thrombosis and necrosis.

Key to the table

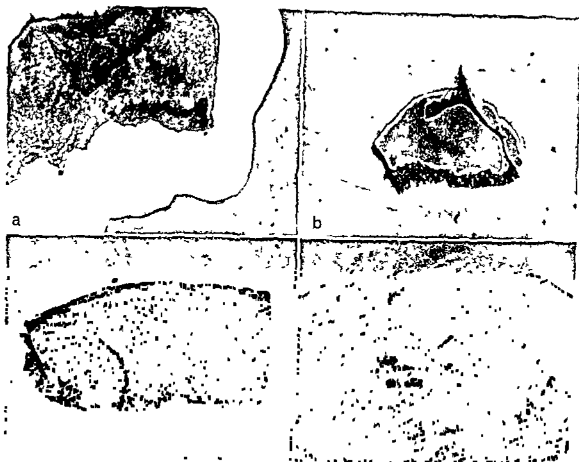
	normal fibrinolytic activity
↓	slight decrease in fibrinolytic activity, i e few active sites in the renal cortex within 120 min incubation.
↓↓	intermediary decrease in fibrinolytic activity, i e no active sites in the renal cortex and few active sites in the renal medulla within 120 min
↓↓↓	marked decrease in fibrinolytic activity i e no fibrinolysis at all in the cortex and medulla within 120 min incubation
+	thrombosis in less than 10 per cent of the glomeruli or blood vessels of the section patchy cortical necrosis
+ +	thrombosis of 10–50 per cent of the glomeruli or blood vessels of the section subcapsular cortical necrosis
+ + +	thrombosis in more than 50 per cent of the glomeruli or blood vessels of the section necrosis of entire cortex and part of the outer medulla
+ + + +	necrosis of the entire renal transplant

DISCUSSION

The study has demonstrated a close correlation between the occurrence of graft thrombosis and decreased fibrinolytic activity. All six cases with thrombosis of intrarenal blood vessels had decreased fibrinolytic activity except one, rabbit number 291 biopsies taken 1, 2 and 3 days after allotransplantation showed a moderate decrease in fibrinolytic activity, but the graft removed 4 days after transplantation showed a fibrinolytic activity within the range of the normal, thrombosis of graft vessels was present in the 3rd day biopsy and in the specimen from the graft removed on the 4th day after transplantation.

The decrease in fibrinolytic activity of the renal graft usually preceded the trombosis. In specimens with massive thrombosis (380, 281, 285), fibrinolytic activity was almost extinct. In the clamped non transplanted kidneys the loss of fibrinolytic activity as measured by the fibrin slide technique preceded thrombosis which was not evident until the 2nd day after clamping. Again, there was a tendency towards a steady decrease in fibrinolytic activity with time after clamping, the fibrinolytic activity of the 2nd day specimen being definitely lowered in one of the cases.

Other studies, too, have demonstrated a relation between thrombosis and decreased fibrinolytic activity. Thrombosis induced in



Decrease in fibrinolytic activity of a renal allograft (rabbit no 285), fibrin slide technique, 120 min incubation, × 12

A The normal, non-transplanted kidney of the rabbit Large zone of lysis in the stained fibrin film extending beyond entire section and causing disruption of the tissue

B Biopsy of renal allograft 1 day after transplantation Moderate zone of lysis related to outer zone of medulla and inner parts of cortex

C 3rd day biopsy and

D Graftectomy specimen, removed on the 4th day, showing *no fibrinolysis at all*

rat femoral vein by injection of thrombin, serum, or sodium morrhuate into ligated venous segments was followed by rapid thrombolysis related to fibrinolytically active sites along the endothelium in thrombin and serum injected animals Thrombolysis was delayed in sodium morrhuate treated animals in whom endothelial fibrinolytic activity was destroyed (*Kwaan & Astrup* 1965) In rabbits infused with thrombin concomittant treatment *with inhibitors of fibrinolysis* (epsilon aminocaproic acid) (*Margaretten & McKay* 1964, *Bellerset al* 1967) produced renal microthrombosis In autopsy studies *Todd & Nunn* (1967) found fibrinolytic activity in the intima of thrombosed veins, on the surface of complete loosened venous thrombi, and on the surface of pulmonary thromboemboli The observations suggested that the fibrinolytic activity of venous endothelium might be responsible for the detachment of emboli In human subjects suffering from venous thrombosis *Pandolfi et al* (1969) found the veins less active than those

from normal controls Fibrinolytic activity in veins occluded by massive thrombi was extremely low

The data from previous and present own studies clearly demonstrate the role of fibrinolytic enzymes of the vascular wall in thrombosis Compared with normal controls thrombosed blood vessels have low fibrinolytic activity Observations from serial biopsies in this study of renal transplantation in rabbits show that decrease in fibrinolytic activity generally precedes thrombosis This might indicate that the plasminogen activator is inhibited or consumed during attempted thrombolysis If the fibrinolytic capacity is overwhelmed, fibrin deposition occurs and thrombi may be formed and persist in the blood vessel

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Fibrinogen preparations used in the fibrin slide technique was kindly provided by dr phil *Tage Astrup* director of research The James F Mitchell Foundation Institute for Medical Research, Washington D C

The study has been supported by grants from the *Danish Medical Research Council*

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Acta path microbiol scand Section A 80, 659 664, 1972

THE OCCURRENCE AND SIGNIFICANCE OF ABNORMAL BILE DUCT EPITHELIUM IN CIRRHOSIS

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Revision of a biopsy series of 497 consecutive cases of cirrhosis revealed 31 with abnormal bile duct epithelium, corresponding to 7 per cent It is rendered probable on the basis of morphological, clinical, biochemical, and immunological findings, that this group of crypto genic cirrhosis has developed from chronic aggressive hepatitis It has previously been shown, that chronic aggressive hepatitis with abnormal bile duct epithelium more quickly develops cirrhosis than chronic aggressive hepatitis without abnormal bile duct epithelium Contrary to this finding cirrhosis with abnormal bile duct epithelium does not, during the rather short period of observation in our material (27 months) exhibit a course, that deviates from other cases of cirrhosis, as the death rate is not different from that of a larger material of cirrhosis

Recently *Christoffersen et al* (1) have demonstrated abnormal bile duct epithelium in 21 cases of a consecutive series of 57 patients exhibiting chronic aggressive hepatitis

No morphological (apart from the abnormal epithelium) or biochemical differences between the two groups were demonstrated There was a *significantly higher incidence of* organ non specific antinuclear factors in the group with abnormal epithelium than in the group without

A morphological follow up showed a significantly more frequent development of cirrhosis in the group with abnormal biliary epithelium

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The purpose of this work has been to investigate how frequently abnormal bile duct epithelium is encountered in a consecutive series of biopsies with cirrhosis and further to investigate which morphological, bio chemical and immunological changes are found in the group of cirrhosis with abnormal *bile duct epithelium*

The purpose has further been to make an examination of the prognostic value of ab normal epithelium by comparing the mortality of this group with the mortality of a large mixed cirrhosis material

MATERIAL AND METHODS

The material consists of 31 percutaneous liver biopsies (from 31 patients) all exhibiting cirrhosis, and all containing abnormal bile duct epithelium Figs 1 and 2)

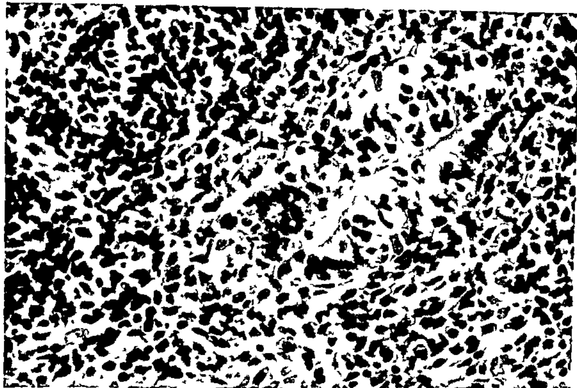


Fig 1 High magnification of bile duct with abnormal epithelium The epithelium is multilayered and the cells enlarged with ill defined cell boundaries pycnotic nuclei and finely granular cytoplasm with a few vacuoles *Enlarged from* $\times$ *400*

The biopsies have been selected as consecutive biopsies from a total of 3000 percutaneous liver biopsies received at the Pathological Anatomical Institute Kommunehospitalet from seven medical departments in Copenhagen in the period December 1965–May 1970

The methods for tecnical procedures and assessment of the biopsies have been described in a previous paper (1)

Further the biopsies have been assessed as re s the size of the nodules—regular or irregular —and with regard to the existence of areas with a partly preserved lobular architecture (4)

In addition clinical biochemical and immunological data have been gathered for 29 out of the 31 patients In two cases the case records were not available The methods used are the same as previously described (1)

A comparison between the death rate of this material's 31 patients and of a larger mixed cirrhosis material (3) has been performed

On calculating the death rate for the 31 patients in question 1st January 1971 has been used as dead line All patients who have not had a follow up examination at this point have been traced through the National Registration office For the patients who have died either an autopsy record or a death certificate is available The death rate for a group of patients is expressed by $h = \frac{x}{n}$ where x is the number of deaths and n is the total sum of months of observation in the group The significance of the difference between the death rates h_1 and h_2 of two groups were estimated by the u value calculated as

$$u = (h_1^* - h_2^*)/\sqrt{h(1-h)} \times \sqrt{1/n_1 + 1/n_2}$$

where $h_1 > h_2$ $h_1^* = h_1 - (1/2n_1)$ $h_2^* = h_2 + (1/2n_2)$ and $h = (x_1 + x_2)/(n_1 + n_2)$

RESULTS

Morphological Findings

Out of a total of 497 biopsies with cirrhosis 31 were found with abnormal *bile duct* epithelium corresponding to 7 per cent

The histological changes in the affected bile duct are exactly as previously described in chronic aggressive hepatitis (1) and affects segments of medium sized *bile ducts*

with a central position in the connective tissue areas corresponding to the original portal areas.

The main histologic changes are multilayered epithelium with swollen frequently pale vacuolized cells frequently with ill defined cellular limits and karyopyknosis. The lumen may contain cellular debris and/or mucin and may be partly obliterated.

In the vicinity there is a considerable infiltration with lymphocytes, histiocytes and plasma cells often in close connection to the affected part of the biliary tree and in some cases germinal centres are observed.

The morphologic criteria we have used for the diagnosis of cirrhosis are *significant fibrosis* and typical nodules of regeneration.

In 30 of the cases the architecture of the liver tissue is partly preserved in some parts of the biopsies, here presenting a picture similar to chronic aggressive hepatitis. The size of the nodules varies. Their diameter may be found to be from much less than the diameter of a normal lobule and up to several centimeters. The nodules are rounded and with irregularly arranged liver cell plates with marked variation in width.

A summary of the changes in the *parenchyma and in the connective tissue* are shown in Table 1.

In the *parenchyma* focal necroses are always present although in a varying number, just as a moderate to severe variation in the size of liver cells and liver cell nuclei are found. Acidophilic bodies and focal Kupffer cell proliferation are always evident.

Confluent necroses are found in five of the biopsies (16 per cent). As a rule they are small and appear in both areas with partly preserved architecture and in regeneration nodules. A little less than one half of the cases (13) present passive septa, most often only one or very few per biopsy. Four of the five biopsies with confluent necroses contain

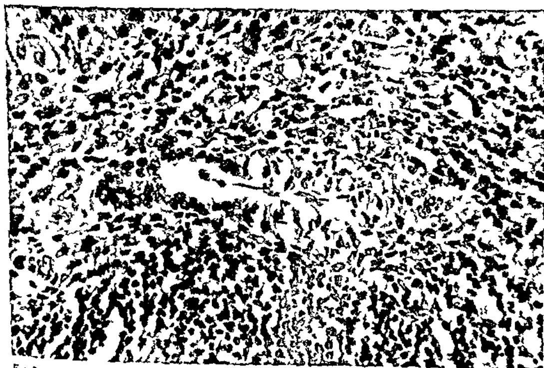


Fig 2 Another example of bile duct with abnormal epithelium. Half of the circumference shows normal epithelium, the other half a picture similar to Fig 1, but with more pronounced vacuolization of the cells. Enlarged from × 250

passive septa as well, whereas only one third (4/13) of the biopsies with passive septa at the same time show confluent necroses

Active septa are unmistakable in all biopsies, and in most cases one finds many diffusely distributed throughout the biopsy

TABLE 1 *Number of Biopsies with the Following Changes in Parenchyma and Connective Tissue in the Material (31 Biopsies with Atypical Bile Duct Epithelium and Cirrhosis)*

Parenchymal changes		+	+ +	+ + +
Focal necroses	31	20	10	1
Confluent necroses	5	2	3	–
Passive septa	13	8	4	1
Active septa	31	8	20	3
Piece meal necroses	31	7	22	2
Acidophilic bodies	31	31	–	
Variation of cells and nuclei	31	9	21	1
Fatty change	8	8		–
Kupffer cell proliferation	31	13	18	–
Adenomatous proliferation	11	10	1	
Cholestasis	5	5		
Parenchymal inflammation	25	25		
Lipofuscin in liver cells	31	31		
Iron in liver cells	0			
Iron in Kupffer cells	0			
Connective tissue changes				
Fibroses	31	1	25	5
Inflammation	31		4	27
Bile duct proliferation	31	12	15	4
Iron in portal macrophages	1	1		
Germinal centres	7	7		
Periductal fibroses	1	1		

In the periportal areas and in peripheral parts of the regeneration nodules one most often finds many and crowded piece meal necroses and in close relation to these many plasma cells

Moderate or severe steatosis has never been seen but in approximately one fourth (8/31) we found lipid vacuoles in a few liver cells

Cholestasis is seen in approximately one sixth (5/31) and adenomatous liver cell proliferation in approximately one third (11/31)

In addition to the inflammatory changes which are seen in relation to piece meal necroses and active septa a slight degree of parenchymal inflammation is seen in most cases (25/31) Plasma cells are found in all cases with parenchymal inflammation

All biopsies contain lipofuscin in liver cells, whereas neither Kupffer cells nor liver cells contain iron pigment

Connective tissue All biopsies exhibit fibrosis and in nearly all cases there is a moderate or severe fibrosis The increase in the amount of connective tissue varies from area to area in the single biopsy, and both broad and slender septa are seen

In all cases an extensive, most often moderate to severe, infiltration with lymphocytes plasma cells and histiocytes is demonstrated in the connective tissue septa Especially many plasma cells are seen in the periphery of the septa in close connection to the parenchyma Apart from a higher frequency of germinal centers in close relation to the abnormal bile ducts there are no differences between the quantity and quality of the inflammatory reaction between the connective tissue areas in the vicinity of the abnormal ducts and the other connective tissue areas

Iron pigment and periductal fibrosis are only demonstrated in one biopsy

There is bile duct proliferation in all biopsies Abnormal bile duct epithelium of the type in question is not to be found in the proliferating bile ducts but in pre existing bile ducts of medium size with a central position in the connective tissue corresponding to the original portal tracts

Clinical Findings

The material comprises 27 women and four men They are all over 45 years of age and the average age is 64 and 71 respectively

The onset of illness was acute (first appearing as a clinical acute hepatitis) in 15 cases insidious in 14 and unknown in two

Biochemical Findings

The average values for some conventional liver tests can be seen in Table 2

Most of the patients have both hypoalbuminaemia (21 (72 per cent)) and hypergammaglobulinaemia (25 (86 per cent))

TABLE 2 *Average of Routine Liver Tests*

Serum bilirubin (<1 0 mg/100 ml)	2 0 (0 2 10 1)
Serum GO Transaminase (<34 U/L)	241 (29–648)
Alkaline phosphatase (<74 U/L)	182 (54–498)

Normal values are given in parenthesis below the name of the laboratory test

Immunological Findings

The results of the immunological findings are seen from Table 3 A high incidence of organ non specific anti nuclear factors (71 per cent) and of smooth muscle antibody (67 per cent) was seen In three of the four cases the SMA were of the IgG class and in the last one the serum contained IgM antibodies

Only one out of seven patients' sera tested showed mitochondrial antibodies and one contained antibodies to thyroid cytoplasm No sera stained gastric parietal cells

With respect to identification of Australian antigen none of the seven available sera showed presence of the antigen

TABLE 3 *Results of Immunological Tests (ANF = anti nuclear factors, SMA = smooth muscle antibody TCA = thyroid cytoplasmic antibody PCA – parietal cell antibody, MA – mitochondrial antibody Au ag = Australian antigen)*

Test	% positive in 50 controls	Number of patients examined	Number of patients with positive sera
ANF	2 %	7	5 (71 %)
SMA	8 %	6	4 (67 %)
TCA	6 %	7	1 (14 %)
PCA	4 %	7	0
MA	2 %	7	1 (14 %)
Au-ag	0 %	7	0

Follow up

The period of observation is between 2 and 63 months with an average of 27 months

In the period of observation, 9 patients died The causes of death for the 9 patients are shown in table 4 The death rate is 1 2 per cent, and in order to examine the prognostic value of abnormal bile duct epithelium, if any, a comparison with the mortality of a large mixed cirrhosis material was made

TABLE 4 *Causes of Death for Nine Patients*

	Number of patients
Coma hepaticum	7
Encephalitis	1
Cardio-vascular disease	1
Total	9

As the age composition, the average period of observation, and the criteria for arriving at a diagnosis of cirrhosis are exactly the same in our material and in the material presented in the *Lancet, January 1969* (3) comprising 334 patients with cirrhosis from the Copenhagen Study Group for Liver Disease, we believe, that a comparison is permissible

The death rate in the large cirrhosis material is 1 8 per cent There is no statistically significant difference between this and death rate in our material

DISCUSSION

As it appears from our results abnormal bile duct epithelium is found in 31 out of 497 cases of cirrhosis It is surprising that 7 per cent of our material of cirrhosis contains abnormal bile duct epithelium when only a few cases previously have been described but we believe that there is no doubt that the expensive use of serial sections is the reason for this difference, as the changes as mentioned are segmental

Abnormal bile duct epithelium has previously very frequently been described in chronic aggressive hepatitis (1) and follow-up investigation showed, that this type of chronic aggressive hepatitis frequently and quickly led to a manifest cirrhosis

It is therefore of interest to analyse those cases of cirrhosis that contain abnormal bile

duct epithelium with regard to whether they can have developed from chronic aggressive hepatitis and whether their prognosis separate them from cirrhosis in general

As it is seen from our results the cirrhosis is in 30 out of the 31 cases of mixed type, i e both small regeneration nodules of the same size or smaller than the original lobules and larger nodules frequently containing remnants of the original lobular architecture possibly as preserved central veins occur

Further in nearly all biopsies in areas with preserved lobular architecture piece meal necroses and active septa with many plasma cells have been demonstrated

The changes in the lobules in the same areas is exactly as previously found in chronic aggressive hepatitis (1)

There are thus a number of morphological changes which render it probable that the majority of the cases of cirrhosis with abnormal bile duct epithelium in question are developed from chronic aggressive hepatitis Also the presence of abnormal bile duct epithelium in itself supports this view as this is found in nearly one half of cases with chronic aggressive hepatitis and only in few cases in other liver affections Furthermore there is nothing in the morphological changes described which opposes this pathogenesis Thus only a few fat vacuoles are seen and no alcoholic hepatitis or Mallory bodies

The considerable preponderance of women in this material also support the above mentioned hypothesis and the same is true for the immunological tests which even though only performed in rather few cases correspond exactly with the findings in patients with chronic aggressive hepatitis

The epithelium changes in the bile ducts and the other morphological findings are different from the findings in primary biliary cirrhosis (2), and neither clinical biochemical nor immunological findings suggest that the cases of cirrhosis presented here are of biliary type

It is therefore our opinion that our results render it probable, that that part of the cryptogenic cirrhoses which contain abnormal bile duct epithelium often possibly always has developed from chronic aggressive hepatitis with abnormal bile duct epithelium

As regards the prognostic significance of the presence of abnormal bile duct epithelium previous investigations have shown that chronic aggressive hepatitis with these changes more quickly develops cirrhosis than chronic aggressive hepatitis without these changes On the contrary cirrhosis with abnormal bile duct epithelium, during the rather short period of observation in our material (27 months), does not seem to have a course which deviates from other cases of cirrhosis as the death rate does not differ from a larger material of cases of cirrhosis (3)

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Acta path microbiol scand Section A 80, 665–670 1972

5-NUCLEOTIDASE ACTIVITY IN THE HUMAN BREAST

Enzyme Histochemical Studies

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Fresh frozen sections of human breast tissue (210 biopsy specimens) are examined for activity of 5 nucleotidase in various benign and malignant pathological conditions 5 nucleotidase is found mainly in the fibroblasts and produce a characteristic stroma response By nickel blocking of 5 nucleotidase it is possible to distinguish the 5 nucleotidase activity from that of the unspecific alkaline phosphatases The technique is described The two enzyme systems are both localized mainly in the fibroblasts, but at different sites

The 5 nucleotidases (5 nucl) are enzymes which catalyse the hydrolysis of nucleotides with resulting production of nucleosides and inorganic phosphate They are classified as specific alkaline phosphatases (International Union of Biochemistry's classification E C 3 1 3 5)

Reis (11, 12) has undertaken thorough investigations into the mode of action and specificity of 5 nucl *Kaye* (5) and *Ahmed & Reis* (1), among others, have later studied the depressing and accelerating effects of various metals on the enzyme activity

5 nucl is extensively present in many organ systems and is often shown to have a striking resemblance to the unspecific alkaline phosphatases (alk phosph) with regard to location and substrate specificity This fact renders difficult the differentiation between the two enzyme systems

Gomori s metal salt phosphatase technique (2) in *Wachstein & Meisel s* (13) modification is employed for histochemical demonstration of 5 nucl

We studied the 5 nucl activity in human breast tissue showing benign and malignant pathological changes The objects of these studies were to give a description of the activity and locations of these enzymes in the human breast, and to make out whether these enzymes take part in the stroma response often described in relation to tumour tissue It is stated by *Monis & Rutenburg* (7), *Okamoto* (9), *Murata et al* (8), and *Matsumura* (6), among others that a stroma response often is seen round tumour tissue manifesting itself by accumulation of briskly proliferating fibroblasts with a high alk phosph activity

MATERIAL AND METHODS

Biopsies were undertaken of breast specimens from 210 women admitted to be operated on for tumour of the breast. The histological diagnoses were as follows Fibro-adenomatosis 102 cases fibrosis 9 cases fibro-adenoma 11 cases, carcinoma in situ 6 cases, and carcinoma 82 cases

The tissue specimens, taken during the opera

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Fig 1 Intense activity of 5-nucl in tracts round a fairly large duct in a biopsy specimen showing fibro adenomatosis (magnification × 60)

Fig 2 The corresponding nickel-blocked tissue section Same magnification The reaction product has disappeared, indicating pure 5 nucl activity

Fig 3 Serial sections from the same field, investigated for alk phosph activity Magnification × 60 Activity is only seen over the distribution of the capillaries (marked with arrows) This weak activity is not visible in the nickel blocked tissue section seen in Fig 2, because only intense activity of alk phosph can be visualized at the altered pH value (Fig 2 pH = 7 2, Fig 3 pH = 9 3)

tion in general anaesthesia, were immediately frozen in isopentane/acetone/dry ice (solid CO_2) at —80° C

Fresh-frozen tissue sections were prepared on cryostat (*SLEE*, Pearse) Tissue thickness 8 μ Next the activity of 5-nucl was demonstrated by the following method (*Wachstein & Meisel 1957*) (*13*).

10 ml of 1 25 per cent adenosine 5-phosphate
5 ml of 0 2 M Trisbuffer at pH 7 2
30 ml of 0 2 per cent $Pb(NO_3)_2$

Two portions of incubation medium were prepared To one of these was further added nickel acetate in an amount corresponding to a concentration of 2 millimol in the incubation medium.

Two series of tissue sections were incubated for 20, 30, 60, and 120 minutes at 37° C The sections were postfixed in formalin, followed by counterstaining with haematoxylin

The histochemical demonstration of 5 nucl. activity is complicated by the ability of alk phosph to hydrolyse the substrate used (adenosine 5 phosphate), though the incubation takes place at a pH value (7 2) which is unfavourable to alk phosph. However, addition of nickel to the incubation medium totally inhibits the activity of 5-nucl without influencing alk phosph The difference between the amounts of reaction products in tissue sections incubated with and without addition of nickel, respectively, thus indicates the 5-nucl activity

The activity of alk phosph was demonstrated by means of *Pearse's* (10) method, using α naphthyl phosphate as specific substrate and Fast Red TR as indicator Incubation periods 20 and 30 minutes at 20° C Counterstained in haematoxylin Postfixation was not employed

The material included for all the tests control sections incubated without the specific substrate

RESULTS

Fibro-adenomatosis The epithelium of ductules and ducts as well as cystic formations displayed no 5-nucl activity in any of the biopsy specimens Neither did proliferating epithelium, if present, show any response In the stroma the fibroblasts were often found to present pronounced activity The intensity varied from a weak colour to thorough overcolouring of the tissue components owing to intense enzyme activity Fibroblasts situated in concentric tracts around lobules and fairly large ducts presented particularly high activity (Figs 1 and 2) The capillaries showed



Figs 4–5 and 6 Corresponding serial sections from a biopsy specimen containing carcinoma (Fig 4 demonstration of 5 nucl without nickel blocking Fig 5 same method with nickel blocking Fig 6 demonstration of alk phosph activity Capillaries marked with arrows)

weak to moderate activity, evenly distributed in all tissue regions

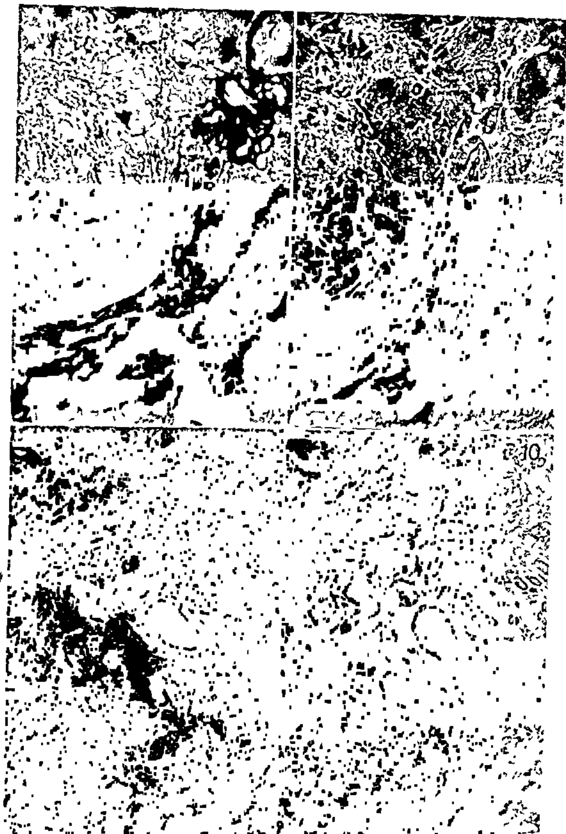
Fibro adenoma and *fibrosis* The epithelial elements, capillaries, and fibroblasts displayed activities identical with those described under fibro adenomatosis

Carcinomas None of the tumour cells showed 5 nucl activity They therefore formed a contrast to the surrounding stroma, in which high activity generally was found to be concentrated in the fibroblasts situated in tracts around the tumour tissue, in some instances splitting this into larger or smaller islets of tumour cells This caused the response product to form a net like structure (Fig 4) The response varied considerably, only some of the stromal fibroblasts showing 5 nucl activity Thus, within the same field, regions showing brisk fibroblast proliferation without 5 nucl activity were often seen side by side with regions displaying intense response (Fig 7)

DISCUSSION

To the author's knowledge only few reports are available on histochemical studies of the 5 nucl activity in the human breast *Wachstein et al* (14) stated in a brief report that 5 nucl activity may occur in the epithelium of ductules, an observation which has not been borne out in the present investigation On the other hand, Wachstein's more recent statement of a failing activity of the tumour cells, but an intense stroma response (15) is in agreement with our findings Likewise, alk phosph often effects an intense stroma response (6 7, 8 9) Comparative studies of the intensities and sites of these two enzyme systems are therefore important

Jensen (3) and *Jensen* & *Schiodt* (4) reported that they often found a stroma-response in the stroma round breast carcinomas represented by fibroblasts with high activity of alk phosph This stroma response was however, no constant phenomenon In several cases brisk fibroblast proliferation was seen in the stroma, but no alk phosph activity Furthermore cases showing high and low o absent activity of alk phosph might occur within the same field of the microscope This variation of the sites of the enzyme activities was the author's direct inspiration to the present investigation from a desire to clarify whether other possibly specific phosphatases might disclose activity in the fibroblasts showing no alk phosph activity



All the biopsy specimens were tested for activity of alk phosph and 5 nucl By performing the tests on serial sections it was possible to compare the enzyme activity of identical structures

The activities of 5 nucl and alk phosph were not localized in the same fibroblasts, though a certain overlapping took place. In the presense of carcinoma, both responses were most often intense, but occurred at different sites In some places, 5 nucl activity was seen at the sites of alk phosph In addition, activity was found in a great number of fibroblasts containing no alk phosph The 5 nucl response was thus observed to be far more pronounced

Biopsy of tissue with carcinoma surrounded by zones presenting brisk fibroblast proliferation, but no alk phosph activity (Fig 4) afforded further evidence of the different sites of the two enzyme systems Demonstration of the 5 nucl activity often disclosed an intense response of these enzymes in the fibroblasts which displayed no activity of alk phosph The blocking with nickel caused the reaction product to disappear completely, indicating that the activity was due to 5 nucl alone (Fig 5) In the biopsy specimens presenting activity of both 5 nucl and alk phosph the nickel blocking method likewise permitted a differentiation of the two enzyme systems (Figs 7–8 and 9–10)

Figs 7–8 and 9 10 Tissue sections where an intense activity of alk phosph yields reaction in the nickel blocked tissue sections (Magnification × 25) Fig 8 shows tissue sections in which the 5 nucl activity has been blocked by nickel Reaction product provoked by alk phosph is seen at the bottom Fig 7 illustrates corresponding tissue sections incubated without nickel blocking Reaction product is seen partly localized within the corresponding region in Fig 8 and partly extending as continuous tracts obliquely upwards through the field of the microscope Above to the right a net like structure is seen The pictures illustrate the different sites of 5 nucl and alk phosph Similar conditions are seen in Figs 9 10 (Fig 9 without nickel and Fig 10 with nickel blocking) Magnification × 25

CONCLUSION

In human breast tissue the epithelial elements showed no 5 nucl activity The capillaries displayed weak to moderate reaction The picture was dominated by the activity of the fibroblasts, which was found to vary—being, however, most often intense The location was irregular, not all fibroblasts presenting 5 nucl activity By comparing the sites of alk phosph and 5 nucl in the fibroblasts, the 5 nucl activity was found to be localized mainly within the regions showing no alk phosph activity

The staining technique employed including blocking of the 5 nucl activity with nickel, permits differentiation of the two enzyme systems

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The work was aided by grants from *Daell Fonden* and *Landsforeningen til Kræftens Bekæmpelse*

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Acta path. microbiol scand Section A 80, 671 686, 1972

RENAL MORPHOLOGY IN BURNED RATS

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The renal structure in rats subjected to a 14 per cent, third degree sublethal thermal injury was studied by light and electron microscopy Haemolysis with resultant haemoglobinuria was found to be associated with the presence of haemoglobin like material in Bowman s spaces, tubular lumina and in the tubular cells The findings indicated that haemoglobin was absorbed by endocytosis and was transported to, and digested within, the lysosomes of the tubule cells in the same way as after intravenous injection of homologous haemoglobin There were no significant morphological signs of tubular degeneration or necrosis, and no intravascular fibrin thrombi were encountered at any of the post burn intervals studied The findings are discussed with particular reference to the changes in renal function following a burn

The cause of renal failure following trauma is still largely unknown, although various pathogenic theories have been proposed Among such mechanisms early attention was paid to tubular degeneration and necrosis (*Bywaters & Dible* 1942, *Lucké* 1946, *Oliver et al* 1951) and cast formation due to excretion of haemoglobin and myoglobin with resultant tubular obstruction (*Oliver* 1945, *Myers et al* 1966) At present most evidence favors reduced glomerular filtration as the major pathogenetic mechanism (*Bull et al* 1950 *Graber & Sevitt* 1959, *Hollenberg et al* 1968) but the cause of this reduction is not settled

After various forms of trauma in man, including burns, disseminated intravascular coagulation has been shown to occur and it has been suggested that intravascular coagulation in the kidneys could be the cause of posttraumatic renal failure (*McKay* 1965, *Hardaway* 1966)

This combined light and electron microscopic study was undertaken to study the renal morphological changes and the possible occurrence of intravascular fibrin deposition in the kidneys in burned rats Since haemolysis is a conspicuous sequela of burn injuries (*Bhargava & Kumar* 1969), a study was made to determine whether or not peroxidative activity was present at the fine structural level in the kidneys Various modes of fixation were employed in order to facilitate correct interpretation of the morphological alterations

MATERIAL AND METHODS

Experimental animals 66 female Sprague Dawley rats weighing 200±5 g (Anticimex Farm, Stockholm) were used The animals were allowed free access to tap water and food (Ewos rat pellets)

Thermal injury The method has been described in detail elsewhere (*Rammer* 1972a) The rat was immersed in water at 90° C for 20 seconds giving a third degree sub-lethal scald, covering 14 per cent of the body surface

Haemolysis Groups of 3 rats were killed at various intervals post burn for determination of haemoglobin in serum Five rats injected with heparin (Vitrum), 200 IU/100 g body weight in 0 5 ml saline, and five rats injected with saline,

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were compared 30 minutes post burn The haemoglobin excretion in the urine was studied *in rats* kept in metabolic cages where *the collecting tubes* were changed every 4 hours For that purpose 7 rats were pretreated with heparin as above and 10 rats with saline Haemoglobin in urine and serum was determined by the cyanmethaemoglobin method, using Acculute® pellets (Ortho Diagnostics) 0 2 ml of serum or urine was added to 4 ml of the reagent, serum or urine from control rats being used as blank

Preparation of kidney tissue for morphological studies At different intervals after the burn (5, 15, 30, 60 minutes and 2, 4, 8, 12 and 24 hours) 2 animals were lightly anaesthetized by an intraperitoneal injection of Nembutal® (Abbot) One rat at each interval was used for perfusion fixation of the kidneys *in vivo* The abdomen was opened by a midline incision, the coeliac and superior mesenteric arteries were ligated and a loose ligature was applied around the aorta immediately below the diaphragm An 18 gauge needle, previously filled with saline and attached to a syringe containing 10 ml of saline, was inserted into the aorta at the bifurcation The inferior vena cava was then opened widely, the aortic ligature was tied and perfusion was started *manually* with saline and continued with fixative, under a pressure of 100 cm water for 5 minutes As a sign of satisfactory perfusion, the kidneys immediately turned yellowish white and became rubbery hard

The fixative used was 1 5 per cent glutaraldehyde solution in Sorensen's phosphate buffer (pH 7 4, ionic strength 0 15) The kidneys were removed, and small cubes of the tissue were immersed in the same fixative at 4° C for 24 hours The pieces were then stored in the phosphate buffer at 4° C pending use within 7 days

In the other rats at the different intervals post-burn the right renal cortex was fixed *in vivo* by dripping 2 per cent *s*-collidine buffered OsO_4 (pH 7 4) *onto* the surface for 15 or 20 minutes (*Ericsson* 1964) The left kidney was removed for immersion fixation of ~ 1 mm thick slices from different parts of the parenchyma (outer and inner cortex, outer and inner medulla) in the buffered OsO_4 or glutaraldehyde solutions

Kidneys fixed by perfusion with glutaraldehyde were subjected to light and electron microscopic studies and to examination for peroxidative activity at the fine structural level For light microscopy paraffin sections were stained with haematoxylin eosin and with Mallory's phosphotungstic acid haematoxylin (PTAH) stain for the demonstration of fibrin For electron microscopy cubes of tissue of a thickness of ~ 1 mm from the cortex and the medulla were immersed in 2 per cent *s*-collidine buffered OsO_4 (pH 7 4) at +4° C for 2–12 *hours and* were subsequently dehydrated in ethyl alcohol and propylene oxide and embedded in Epon epoxyresin Staining "en bloc" with uranyl acetate in 100 per cent ethyl alcohol was performed Thin sections were cut on an LKB Ultrotome, stained with lead citrate and examined in a Siemens Elmiskop I electron microscope

For the histochemical demonstration of peroxidative activity (haemoglobin and related substances) (*Smith & Beck* 1967), ~ 50 µ thick frozen sections of glutaraldehyde perfused, DMSO treated (*Helminen & Ericsson* 1970) tissues, were incubated at room temperature for 20 minutes in 5 mg of 3 3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) dissolved in 10 ml of TRIS buffer, pH 7 6 (*Schneeberger-Keeley & Karnovsky* 1968) 0 1 ml of 1 per cent H_2O_2 was then added the solution gently stirred and incubation continued for a further 20 minutes The sections were then washed twice in 0 1 M cacodylate buffer (pH 7 4) and post fixed in the buffered OsO_4

Dehydration and further treatment for electron microscopy were performed as described above

Following drip fixation, the renal capsule with the adjacent ~ 0 5 mm thick well fixed zone of outer cortex was rapidly dissected out under buffered OsO_4 and immersed in the OsO_4 solution for 2 hours at +4°C The tissue was then processed for light and electron microscopy as described above

Tissues fixed by immersion in glutaraldehyde or OsO_4 were handled in the same fashion as those fixed by perfusion and subjected to light and electron microscopic examination

For orientation in the Epon blocks and selection of appropriate areas for electron microscopic observations ~ 1 µ thick sections stained with alkaline toluidine blue were studied in the light microscope

RESULTS

1 *Haemolysis* The serum haemoglobin concentration at different intervals is shown in Fig 1 Maximal values of about 400 mg/100 ml were noted immediately post burn and the concentration then rapidly decreased After 2 hours no haemoglobin was detected in the serum Pretreatment with heparin did not influence the degree of haemolysis 30 minutes post-burn During the first four hours the haemoglobin excretion in the urine was 1 08±1 00 mg (mean±S D) in animals pretreated with saline and 1 56±1 00 mg (mean±S D) in heparin pretreated animals, a difference that was not statistically significant In the urine samples

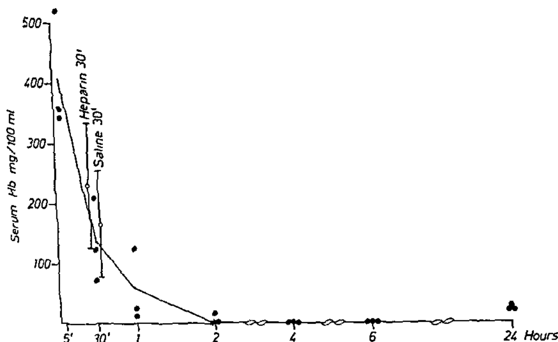


Fig 1 Diagram illustrating serum haemoglobin concentration at various intervals postburn At the 30 minute interval 5 heparin and 5 saline pretreated rats are compared (mean ± S D)

taken later, no haemoglobin was found The red colour of the urine persisted after centrifugation, and microscopic examination of the urinary sediment showed numerous casts but no red cells

2 *Glomeruli* No material with the staining characteristics of fibrin or with periodical striation at a frequency of about 200 Å by electron microscopy (*Margaretten et al* 1967) was found, therefore, no evidence of the presence of fibrin in glomerular capillaries was obtained (Figs 2, 3 and 11) At early intervals (15 minutes to 2 hours) after the burn the capillary lumina contained a finely granular, diffusely distributed substance, which was positive when tested for the occurrence of peroxidative activity ('haemoglobin like material') The material was believed to represent haemoglobin containing plasma In Bowman's spaces similar although less concentrated material was observed There were no alterations in the fine structure of the endothelial and epithelial cells or of the glomerular basement membrane

3 *Proximal convoluted tubules* The fine structure at different intervals postburn is illustrated in Figs 4 10 and 13–16 No changes in the nuclei, mitochondria, endoplasmic reticulum, microbodies, lateral and basilar plasma membranes, Golgi regions or the cytoplasmic ground substance were observed Cellular autophagy was not increased As examplified in Figs 11 and 12 the tubular lumina were patent Between the 15-minute and 2 hour intervals the lumina contained haemoglobin like material of varying concentration but no crystalline material was seen Starting at 30 minutes and continuing until 8 hours after the burn, the haemoglobin like material accumulating around the microvilli of the brush border was taken up into the cells by pinching off of vesicles ('apical vesicles") and vacuoles ('apical vacuoles") from the basilar invaginations of the plasma membrane constituting the microvilli (Figs 4 and 5) This material became concentrated in electron opaque "absorption droplets", which were most numerous 2 to 4 hours after the burn (Figs 6–9),

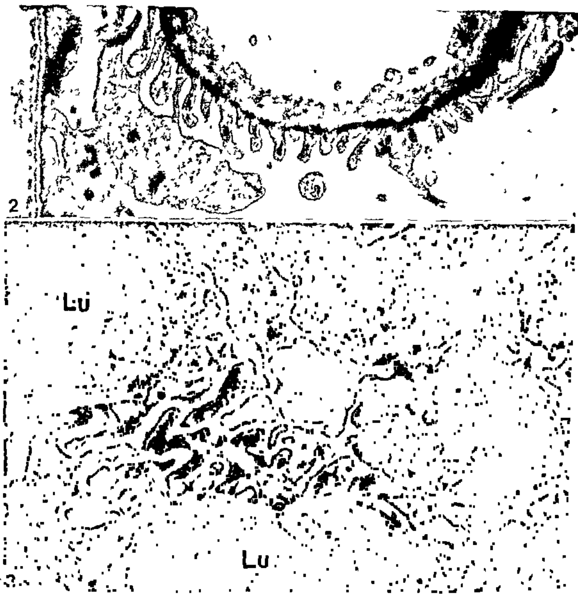


Fig 2 15 min, persfusion fixation, glomerulus The glomerular capillaries have a normal appearance × *12,500*

Fig 3 60 min immersion fixation (OsO_4), glomerulus A finely granular, diffusely distributed material, presumed to be plasma-containing haemoglobin, is present in the capillary lumina (Lu) A similar, though less concentrated substance is seen in Bowman's space (arrows) Otherwise, the structure is unaltered × 15,000

Fig 4 30 min, perfusion fixation, incubation in benzidine–H_2O_2 reagent, proximal convoluted tubule A reaction product signifying the presence of haemoglobin is deposited in apical vesicles (Av) and in a large cytoplasmic body (B), and also extracellularly between the microvilli in the brush border (arrows) × 40,000

Fig 5 60 min, drip fixation, proximal convoluted tubule Many apically located droplets (Dr) with an electron dense, homogeneous content are present The apical vacuoles (AV) are irregular in shape and contain a finely granular material *BB*, brush border × 17,500



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at 8 hours they were infrequent Between 2 and 12 hours many lysosomes appeared to accumulate haemoglobin like material (Figs 9, 10 and 13), and starting at the 4-hour interval some lysosomes contained granules, up to 100 Å in diameter (Figs 10 and 13–15), resembling the iron positive material demonstrated in the tubular lysosomes of haemoglobin treated animals (*Ericsson* 1965 b) At 24 hours all the cells appeared normal, with the exception that some lysosomes encompassed the granular, haemosiderin-like substance Some cells showed a normal structure as early as 8 hours post burn (Fig 16)

4 *Pars recta of proximal tubules* Changes similar to those seen in the proximal convoluted tubules were observed, although the absorption droplets were smaller and less abundant The lumina were always patent

5 *Henle's loops* Most of the loops showed no noteworthy changes throughout the experiment (cf Fig 12), however, occasional homogeneous, peroxidase positive casts were encountered, usually between 2 and 8 hours after the burn The lumina were patent at all intervals

6 *Distal convoluted tubules and collecting ducts* Light microscopy revealed that occasional convoluted tubules and cortical ducts contained haemoglobin like casts with a crystalline appearance, notably 8 and 12 hours after the injury (Fig 19) In such tubules, apparent mechanical damage with rupture of apical portions of the cells was often seen both by light and electron microscopy Some cells appeared to have absorbed small amounts of haemoglobin like material (Fig 17) Otherwise neither the distal convoluted tubules nor the collecting ducts showed any noticeable change (Fig 18)

7 *Renal vessels and juxtaglomerular apparatus* No thrombi were seen and no alterations were observed in the vessel walls The granulation of the juxta glomerular cells was not investigated in detail

DISCUSSION

Immediately after the burn, free haemoglobin appeared in serum but after 2 hours it was no longer detectable The prompt start of the haemolysis after the burn suggests that it was the result of thermal injury to the blood passing through the heated part of the skin (*Bhargava* & *Kumar* 1969)

The main abnormal finding in this study of the renal morphology in burned rats was *the occurrence of haemoglobin like, proteinaceous material in glomerular capillaries, Bowman's spaces, tubular lumina and within the proximal tubular epithelial cells* The abundance of this material shortly after the burn is explicable by an increased permeability of the glomerular capillary walls,

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Fig 6 2 hrs, drip fixation, proximal convoluted tubule AV, apical vacuoles, BB brush border, Dr, apically located droplets, Lu, empty tubular lumen. × 12,000

Fig 7 4 hrs, drip fixation, proximal convoluted tubule AV, *apical vacuoles*, BB, brush border, Dr, apically located droplets, m mitochondria with normal appearance × 15 000

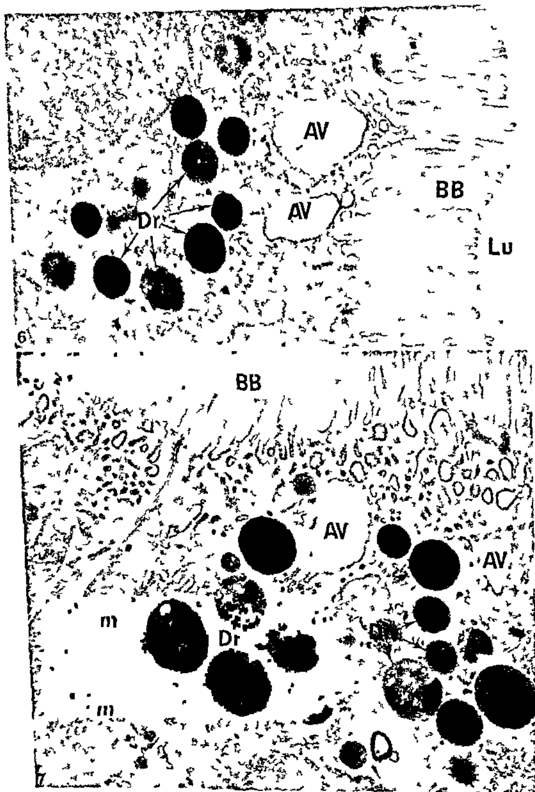
Fig 8 4 hrs drip fixation, proximal convoluted tubule AV, apical vacuole, BB brush border, Dr droplet, G, Golgi apparatus, L *unaltered lysosome* × 11,000

Fig 9 4 hrs, *drip fixation, proximal* convoluted tubule AV, apical vacuole, Dr, droplets, L, lysosome, Lm possibly altered lysosomes (with clumps of haemoglobin like material) × 12 500

Fig 10 4 hrs, drip fixation, proximal convoluted tubule Four bodies (Lm) *possibly comprising modified lysosomes are* shown Note the presence of accumulations of extremely electron dense granular material (arrows) in two of the bodies × 12,500

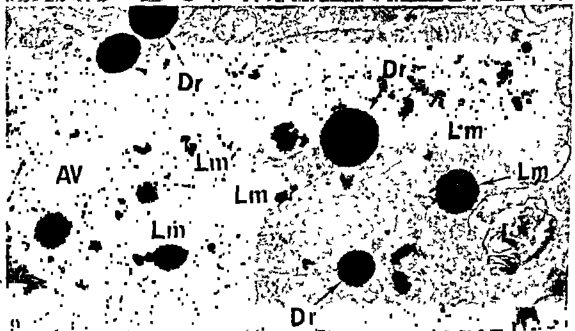
Fig 11 15 min, perfusion fixation *light micrograph of toluidine blue stained* Epon section A glomerulus (Gl), proximal convoluted tubules (PT), a distal tubule (DT), and a collecting *duct* (CD) all appear normal × 600

Fig 12 8 hrs perfusion fixation, *light micro graph of toluidine blue stained* Epon section Proximal convoluted tubules (PT) a distal tubule (DT), and a collecting duct (CD) all show a normal appearance *Inset* shows intact thin loops of *Henle at 4 hrs* × 700



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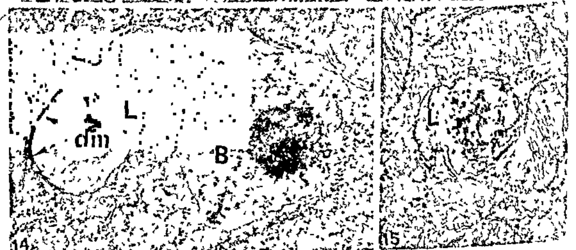

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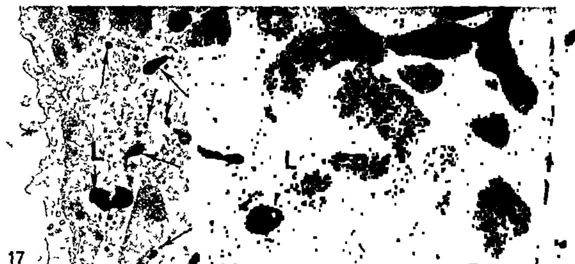

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Fig 19 12 hrs perfusion fixation, light micro graph of toluidine blue stained Epon section (distal convoluted tubule) The lumen is filled with crystals of haemoglobin like material × 1 000

Fig 13 8 hrs drip fixation, proximal convoluted tubule Mid portion of a cell with at least two bodies (Lm) that may represent altered lysosomes AV apical vacuoles Dr, droplets with contents of varying density Note the normal appearance of the mitochondria × 14 500

Fig 14 Same tissue as in Fig 13 An apparant lysosome (L) with peripherally located "dense and membranous material (dm) and an aggregate of granules (*arrow*), as well as a body (B)—either representing a droplet or an altered lysosome—are seen × 21 000

Fig 15 Same tissue as in Figs 13 and 14 A lysosome like body (L) with abundant granules of the type seen in haemosiderin deposits is shown × 31 500

Fig 16 8 hrs drip fixation proximal convoluted tubule *Part of a cell with an appearance indistin*guishable from that in the controls Av autophagic vacuole BM basement membrane L, lysosomes × 13 500

Fig 17 4 hrs perfusion fixation incubation in benzidine H_2O_2 reagent distal convoluted tubule Reaction product is present in apically located vescular or tubular structures (*arrows*) and probably also in lysosomes (L) There are no morphological signs of cell damage × 16 000

Fig 18 8 hrs perfusion fixation, base of distal *convoluted tubule The appearance* is normal × 15 000

which has been shown to occur in patients with severe burns (*Arturson* 1961) As the material was positive with the benzidine technique it appears that it contained haemoglobin, however, it cannot be excluded that part of it represented myoglobin The material was taken up into the "intracellular digestive tract" of the tubular epithelium (endocytosis vesicles and vacuoles, absorption droplets and lysosomes), and the observations indicated that absorption, transport and digestion of the material in the *proximal* tubules occurred in the same fashion as after injection of homologous haemoglobin (*Ericsson* 1965 a, b, *Maunsbach* 1966, *Maunsbach & Neustein* 1968, *Ericsson & Maunsbach* 1972) The formation of crystals (*Ericsson et al* 1969) in the distal portions of the nephron occasionally appeared to exert a direct mechanical effect on the cells It is not clear whether these changes were only preparation artifacts resulting from displacement of the crystals during sectioning or indicated true cellular damage Such damaged tubules were rare and the change is unlikely to have affected the function of the kidneys

Apart from these changes no signs of damage to the tubular epithelium at any level such as rupture of lysosomes, contraction or swelling of mitochondria or increased autophagy were observed Light microscopy on formalin fixed material did not show any tubular damage either at 2 or 3 days postburn The results are in contrast to the findings in burned dogs where degenerative changes in the tubular epithelium have been observed in several studies (*Elrod et al* 1951, *Fergusson et al* 1967) In a parallel study of the renal function in burned rats (*Rammer* 1972 b), a pronounced initial depression of the glomerular filtration rate was found but the tubular function—measured with TmPAH—was unimpaired The burn induced haemolysis with urinary excretion and tubular uptake of haemoglobin like material thus did not cause any significant damage to the tubular epithelium as judged from the functional and morphological studies

Considerable controversy exists regarding

the effect of haemolysis upon the kidneys Injection of glycerol in rats resulted in haemolysis and development of renal necroses whereas injection of homologous haemoglobin resulting in an equivalent plasma haemoglobin concentration did not give rise to tubular cell damage as judged by electron microscopy (*Ericsson et al* 1969, *Ericsson* & *Mostofi* 1969) and by enzyme histochemical methods (*Dallner* & *Ericsson* 1964) It therefore appears that the glycerol induced renal changes were a result of factors other than the occurrence of free haemoglobin In studies of the renal function after infusion of autologous haemolysed blood or a commercial purified human haemoglobin preparation in man *Miller* & *McDonald* (1951) found a depression of inulin and PAH clearances *Jaenike* (1967) observed a reduction of the renal blood flow and inulin clearance during the initial hours after injection of homologous haemoglobin in rats These studies indicate an influence by haemoglobin *per se* on the renal perfusion and such changes in renal function might not be well reflected by morphologically demonstrable alterations

It has long been agreed that the glomeruli show no morphological abnormalities and contain no thrombi in most patients with acute renal failure (*Brun* & *Munck* 1957 *Olsen* & *Skjoldborg* 1967 *Schubert* 1968) However in a small number of posttraumatic patients fibrin thrombi have been found in the renal glomeruli at autopsi especially after burns (*Sevitt* 1956 *Ecles* & *Sevitt* 1967) Recently *Clarkson et al* (1970) found by electron microscopic study of renal biopsies glomerular fibrin deposits in most patients with acute renal failure Light microscopy did not reveal the fibrin in these cases

In the study of the renal function in burned rats (*Rammer* 1972 b) previous heparinization or fibrinolysis inhibition with tranexamic acid was found not to influence the renal functional changes post burn This result is in agreement with the absence of fibrin in the renal vasculature in the burned rats of the present study The frequency of the intervals at which the observations were made and the various fixation methods should exclude the error of intravital and postmortal fibrinolysis At 2 and 3 days post burn light microscopy of formalin fixed material did not reveal any fibrin The result is in agreement with most previous studies of the renal morphology after trauma in experimental animals (*Rollhauser* & *Vogell* 1960 *Hoyle et al* 1969) Only one brief statement of the existence of small amounts of fibrin in the renal vessels in burned rabbits is available (*Hey et al* 1969)

Thus no morphological or functional evidence of persistant renal damage and no signs of fibrin deposition in the kidneys were found in the burned rats This experimental trauma model therefore does not permit conclusions on the importance of fibrin deposition in the kidneys for the development of posttraumatic renal failure

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Supported by grants from the *Swedish Medical Research Council* (Nos B 71–40X 2035–05 and B 72–40 X 2035–06) the *Torsten and Ragnar Soderberg Foundation AB Pharmacia* and the *Medical Faculty University of Uppsala*

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Acta path microbiol scand Section A 80, 687-688, 1972

BRIEF REPORTS

DUAL ORIGIN OF β AMINOISOBUTYRIC ACID, A THYMINE CATABOLITE

Henrik Rist Nielsen, Ernest Borek, Knud Erik Sjolin and Kaare Nyholm

Recently we have found an increased excretion of β aminoisobutyric acid (β-AIB) in urine from patients under treatment for urothelial tumours (*Nielsen et al* 1970, 1971)

β AIB was first isolated and identified in human urine by *Crumpler et al* (1951), and *Fink et al* (1951) *Fink et al* (1951) *and Fink et al* (1956) showed that in rats β AIB may be produced from thymine *Gartler* (1959) demonstrated that the same is the case in man From thymine the production proceeds reversibly to dihydrothymine and β ureidoisobutyric acid, and further irreversibly to β AIB *Kupiecki* & *Coon* (1957) claimed valine to be a precursor of β AIB *Gartler* (1959) (8) and *Armstrong et al* (1963) showed in tolerance tests that the amount of β AIB produced from valine is very small about 10 per cent, compared with that originating from thymine

In healthy persons β AIB is usually excreted in the urine in small amounts (*Evered* 1956, *Soupart* 1959, *Nielsen et al* 1971 *Nielsen* 1972) In some pathological conditions among which are cancer diseases a high urinary excretion of β AIB has been reported This has been discussed in detail earlier (*Nielsen et al* 1971) It is well known that thymine occurs in small intracellular pools of thymine nucleotides and in DNA as well as in tRNA In contradistinction to DNA, tRNA contains only one thymine base per molecule Thymine in DNA is derived from uridine monophosphate by methylation with $N^{5,10}$ methylenetetrahydrofolic acid where the methylene group is derived from formate after the scheme of *Sneider* & *Potter* (1969)

On the other hand it has been shown by *Borek* & *Srinivasan* (1966) and *Srinivasan* & *Borek* (1966) that the methylated bases in tRNA are formed by addition of methyl groups to the preformed macromolecule by specific enzymes with S adenosylmethionine as methyl donor

Received 20 iv 72 from the *Department of Clinical Chemistry Sundby Hospital, DK 2300 Copenhagen S Denmark Department of Microbiology, University of Colorado Medical Center, Denver, Colorado Department of Pathology and Department of Surgery Sundby Hospital, DK 2300 Copenhagen S Denmark*

The purpose of the present experiments was to explore on the basis of this knowledge whether the source of the excreted β AIB is one or both of these thymines

Male adult Sprague Dawley rats were injected intraperitoneally with ^{14}C formic acid (5 mC/mM) and ^{3}H methylmethionine (1 mC/0 0456 mg) respectively The urine was collected in 24 hour samples during the first 96 hours after injection β AIB was isolated from the urine by thin layer chromatography of the dinitrophenyl (DNP) derivatives (*Goedde* & *Brunschede* 1965, *Nielsen et al* 1971, *Nielsen* 1972) The spots containing DNP β AIB were scraped off and counted in a Beckman 250 S liquid scintillation counter All determinations were done on duplicate samples

The excretion of β AIB was less than 0 3 mmol/l, in all rats therefore, the maximum was about 4 μmol/24 h

Table 1 contains the results of our experiments After administration of both formate and methionine, labelled β AIB was isolated Labelling was high the first day but there was a decrease in radioactivity after that.

By using tritiated and not ^{14}C methylmethionine we were able to exclude the possibility *of the* incorporation of radioactivity via the pathway of methionine to formate *through the one* carbon pool This way we can conclude that ^{3}H labeled β AIB was derived from tRNA thymine, since the tritium is exchanged if the $-CH_3$ becomes a source of formate

Therefore, β AIB is derived from both DNA *thymine* and tRNA thymine

So far the excretion of β AIB has been considered to be an expression of DNA turnover, however the demonstration of the dual origin of β AIB indicates a more probable explanation of the high urinary excretion of β AIB, especially in tumour bearing patients since the turnover of DNA is known to be slow compared to the turnover of tRNA

The available data merely document qualitatively the dual origin of β AIB, they do not suffice

TABLE 1 *Comparison of Labelled β Aminoisobutyric Acid in 24 H Urine Samples from Sprague Dawley Rats after Injection of ¹⁴C Formic Acid and ³H Methyl Methionine Respectively*

Injected i p	Rat	1 day β AIB CPM/24 h	2 day β AIB CPM/24 h	3 day β AIB CPM/24 h	4 day β AIB CPM/24 h
^{14}C formic					
acid	A	4,260	1,590	1,890	780
100 μC	B	3,310	210	130	75
^{3}H methyl	C	28 050	5 610	1,570	150
methionine	D	33,790	2 250	1,610	0
400 μC	E	15,850	8,440		0

for a calculation of the relative magnitude of the two sources However, the very rapid diminution of label from the second day to the fourth in the β AIB derived from methionine conforms to the well known high turnover of tRNA This high turnover of tRNA has been observed in urine via other products which stem from tRNA viz pseudouridine and the methylated purines

The authors are indepted to V' *Bremerskov*, M D, Dept Pathology Finsen Institute, Copenhagen and Mrs *Birgit Vingaard* for their valuable technical assistance

This investigation was supported by grants from *Landsforeningen til Kræftens Bekæmpelse (Danish Cancer Society)*

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Acta path microbiol scand Section A 80, 689–690, 1972

DEMONSTRATION OF INSULIN IN AN ISLET CELL PANCREATIC ADENOMA USING AN IMMUNOFLUORESCENT TECHNIQUE

Ole Frokjær Thomsen

It is well known that insulin can be demonstrated in beta-cells of normal pancreatic islets by immunofluorescence, but thus far it has not been possible to demonstrate insulin in neoplastic cells of islet-cell pancreatic adenomas (insulomas) (1, 2 3)

In this paper a report is given of the successfull demonstration of insulin in an insuloma using an immunofluorescence method with Bouin fixed, paraffin embedded material This method has previously been shown to be a sensitive stain for insulin in an immunofluorescence system (*Thomsen* 1971)

Material and Methods

A 57 year old woman was admitted to hospital because of attacks of hypoglycemia during several months Clinically, hypoglycemia accompanied by nervous symptoms could be provoked by starvation during which blood sugar reduction to 28 mg per cent was measured Attacks were releaved by intravenous administration of glucose

On pancreatic arteriography a tumor was demonstrated in the head of the pancreas The tumor was removed, it was round weighed 2000 mg and measured 14 mm in diameter The tumor was surrounded by a thin capsule and on section was quite firm homogeneous yellow red

The tumor was fixed partly in formalin partly in Bouin s fluid (saturated aqueous picric acid formalin and acetic acid 15 5 1) and embedded in paraffin In addition some of the tissue was quick frozen in isopentane at —70° C and stored at that temperature Cryostat cut sections from applicated being a crude anti insulin serum from a guinea pig (insulin binding capacity higher than 1 unit/ml), and the second layer a fluorescein isothiocyanate labelled antiglobulin against guinea pig immunoglobulin, prepared in a rabbit (Nordic Diagnostics) The following were used as control sections a) sections where the first layer applied was a normal guinea pig serum b) sections on which only the fluorescent second layer was applied Paraffin embedding, staining procedure, and microscopic equipment were as described in a previous paper (4)

Results and Comment

Light microscopy Examination of hematoxylin/eosin stained sections showed the tumor to be surrounded by a thin fibrous capsule The tumor was composed of irregular islets and trabeculae of epithelial cells situated in a dense, collagenous stroma with very few cells Scattered calcified, psammoma body like structures were present There were no signs of malignancy

Immunofluorescence microscopy Examination of the paraffin sections revealed a rather strong, apple green fluorescence in the cytoplasm of the epithelial cells The surrounding stroma did not fluoresce (Fig 1 and 2) Inside clusters of fluorescent cells small groups of non fluorescent cells were sometimes present (Fig 2) Adjacent to the tumor were sparse amounts of pancreatic tissue with normal appearing islets, these displayed beta-cell fluorescence (Fig 3)

In the control sections no fluorescence was seen in the epithelial cells of the tumor nor in the surrounding normal islets (Fig 4) Immunofluorescent examination of the frozen sections revealed a very weak fluorescence in the epithelial cells

The specific fluorescence in the epithelial cells, as encountered in the paraffin sections must be taken as an expression of the fact that these cells contained insulin in their cytoplasm, and consequently that the tumor was composed of proliferating insulin bearing beta-cells situated in a dense collagenous stroma

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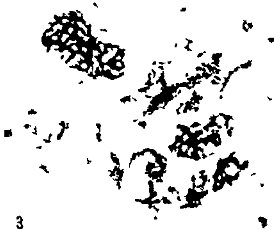
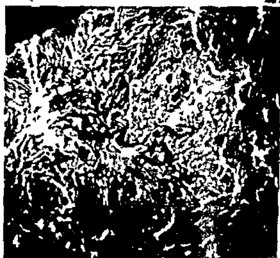



Fig 1 Trabeculae of fluorescent epithelial cells in the insuloma Stain for insulin × 120

Fig 2 Islet of fluorescent epithelial cells in the insuloma The central dark area represents nonfluorescent cells Stain for insulin × 300

Fig 3 Two normal islets in adjacent pancreatic tissue displaying fluorescent beta cells Stain for insulin × 400

Fig 4 Non fluorescent epithelial cells in section stained with a normal guinea pig serum instead of anti insulin serum × 120

This is in agreement with the clinical findings as the patient no longer had attacks of hypoglycemia after removal of the tumor likewise the blood sugar which had been low before operation became normal

The reason why previous attempts to demonstrate insulin in insulomas by immunofluorescence have not succeeded may be due to differences in cytochemical structure or immunological reactivity between neoplastic and normal β cells Moreover the technical procedure might play some role In this case the reaction was evident in the Bouin fixed paraffin embedded tissue whereas as mentioned it was very weak in frozen sections of tissue stored in isopentane at −70° C

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Acta path. microbiol. scand. Section A 80, 691–693, 1972

ANGIOGRAPHY IN DIBUTHYLNITROSAMINE-INDUCED RAT BLADDER TUMOURS

Leif Ekelund, Jan Göthlin and Hans Henrikson

Recently, a method for catheterization of arteries in the rat has been developed (*Ekelund & Olin* 1970) and applied with good results in an angiographic study of dimethylnitrosamine induced renal tumours (*Ekelund & Jonsson* 1971). Tumours of various organs of the rat may be induced by different nitrosamine compounds (*Magee & Barnes* 1959, 1962, *Druckrey et al.* 1964, *Ito et al.* 1969) and the present paper is a preliminary report of an angiographic study of dibuthylnitrosamine-(DBN)—produced urinary bladder tumours.

Material and Methods

Twenty white rats of a Wistar strain, predominantly males, were used. Their weight at the beginning of the experiment was 175 to 200 g. Dibuthylnitrosamine (Eastman—Kodak) was added to the drinking water in the proportion 250 mg DBN in 1000 ml tapwater as described by *Bertram & Craig* 1970. The mean dose of DBN per rat and week was estimated to be 110 mg.

Several rats died early in the experimental period so that angiography was only performed in 5 rats, in 4 of these 4 months after the beginning of the carcinogen administration and in 1 rat after 6 months.

The technical details concerning catheterization have been described previously (*Ekelund & Olin* 1970). Pelvic arteriography was performed with the tip of the catheter in the distal part of the lumbar aorta. Selective catheterization of the internal iliac artery was made in 2 rats, but this resulted in wedge position of the catheter and subsequent spasm, due to the small caliber of the vessel.

Two types of angiographic technique were applied. One was to place the film just below the rat, thus obtaining a single radiogram. The roentgenological data were 0,1 mm focus, 1,3 mAs, 0,05 s 95 kV. The other method was to use a filmchanger for magnification angiography of small animals (*Angantyr & Olin* 1971) and the series was 1 film/s for 6 s. The roentgenological data were 0,3 mm focus, 20 mAs, 0,04 s and 90 kV.

Contrast medium (Isopaque 350, Nyco, Norway) was injected by hand at a suitable rate and industrial roentgenfilm (Structurix, D 4, Agfa Gevaert, Germany) was used for the angiograms.

After angiography the rats were sacrificed and autopsied. The bladders were fixed in 10 per cent neutral formalin, embedded *in toto* in paraffin and cut in semiserial sections. The sections were stained with haematoxylin—eosin and according to van Gieson.

Results

Angiography. In 4 of the 5 rats examined by angiography a bladder tumour could be demonstrated. The largest tumour was found in the rat examined 6 months after the introduction of carcinogen and in this case tumour vessels were obvious on the angiograms (Fig. 1a). In the 4 rats examined after 4 months, a bladder tumour appearing as a contrast defect could be found in 3 cases. In 2 of these cases the tumour was too small to allow demonstration of tumour vessels, but in the third case a few pathological vessels could be seen (Fig. 2a). In 1 case no tumour at all could be detected at angiography.

Pathology. In all the five rats bladder tumours were found, in three of them they were multifocal. The largest tumour from the rat exposed to DBN for six months had a diameter of 15 mm and was occupying almost the entire bladder. Microscopically it was a partially low differentiated squamous cell carcinoma broadly invading the muscle wall and penetrating to the outer surface of the bladder (Fig. 1b). In the other four cases the tumours were five mm or less, papillomatous, often pedunculated (Fig. 2b) with the histological picture of highly differentiated squamous cell carcinoma, infiltrating the lamina propria but not the muscle wall. The adjacent epithelium was often hyperplastic with foci of squamous metaplasia and with oedema of

Received 29.vi.72 from the *Roentgendiagnostic Department (Section for Experimental Roentgenology)* and *Institute of Pathology, University Hospital, Lund, Sweden.*





Fig 1a Pelvic angiography 6 months after introduction of DBN Large tumour occupying almost the entire urinary bladder Tortuos tumour vessels are filled with contrast (→)

Fig 1b Photomicrograph of the squamous cell carcinoma, in some areas of low differentiation, with diffuse infiltration of the bladder wall Haematoxylin erythrosin × 100

the underlying connective tissue Metastases of the tumours were not observed

Discussion

In vivo angiography is a new approach in the study of various experimental tumours in rats The growth of a tumour can be followed and the effect of different types of treatment can be evaluated by this method as it is possible to perform a second catheterization from the contralateral femoral artery and eventually a third examination from the left carotid artery

Our material is still small but nevertheless we want to present it to show that it is possible to study bladder tumours in the rat by angiography

It has been shown that bladder tumours may be induced with high frequency by DBN and in our 5 cases examined by angiography all had developed bladder tumours 4 of which could be diagnosed by radiological examination The optimal time for angiographic investigation seems to be about 6 months after the introduction of carcinogen

All the tumours were histologically squamous cell carcinomas, a finding in accordance with the report of *Bertram & Craig* 1970 who induced bladder tumours in mice with DBN

This work was supported by grants from the *John and Augusta Persson Fund*

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Fig 2a Pelvic angiography 4 months after carcinogen introduction The tumour is seen as a polypous contrast defect in the left part of the bladder Faint contrast filling of tomour vessels

Fig 2b The low power photomicrograph shows the pedunculated tumour that is a *highly differentiated* squamous cell carcinoma with invasion of the stroma Haematoxylinerythrosin × 27

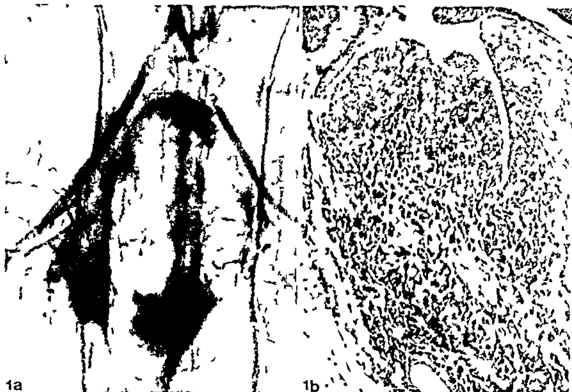


Fig 1a Pelvic angiography 6 months after introduction of DBN Large tumour occupying almost the entire urinary bladder Tortuos tumour vessels are filled with contrast (→)

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S O Emdin & Y Östberg SCANNING ELECTRON MICROSCOPY OF THE GLOMERULAR LESIONS IN MICE WITH AUTO IMMUNE NEPHROPATHY

NZB/NZW F1 hybrid mice develop a disease analogous to systemic lupus erythematosus (SLE) in man with a progressive nephropathy This spontaneous animal disease is an excellent experimental model for studying the evolution of the glomerular lesions Particular attention must be paid to the morphological changes in the podocytes A parallel

wire loop lesion were seen In TEM, fusion and atrophy of the foot processes of the podocytes were observed, as well as a vacuolization of the cyto plasm and nodular thickening of the basal membrane with deposition of an osmiophilic material

By SEM, extensive areas of the glomerular tufts could be studied, allowing a wide range of varying stages of the glomerular destruction to be investigated In the initial stages, when still no light microscopical lesions were observed, fusion and atrophy of the terminal processes of the podocytes were observed In later stages shrinkage and invaginations of the cell membrane of the podocytes occurred, and ultimately the cells got a spongy appearance

On the basis of the sequence of changes it was proposed that the early alterations in the foot processes were a compensatory action of the podocytes for the damaged basal membrane

S R Orell MICROBIOPSY FROM THE URINARY BLADDER MUCOSA WITH MINIMAL TRAUMATIZATION

A method is presented by which small fragments of bladder epithelium—papillomatous or non-exophytic—may be obtained with minimal traumatization The end of an ureteric catheter is cut off at oblique angle and the proximal end is connected tightly via a cannula to a plastic 10 cc syringe The catheter is introduced into the bladder through a resectoscope and directed to the lesion under visual control While the surgeon gently scratches the surface of the lesion with the end of the catheter, the assistant aspirates slowly, allowing only a small volume of fluid to enter the syringe The material obtained + 1 2 drops of albumen is fixed in 1–2 cc Stieve s fluid and centrifuged at 6 000 r p m. The resulting plug of coagulated albumen containing the epithelial fragments is embedded in paraffin sectioned and stained by routine methods

The method involves the following advantages

1 Traumatization is limited to insignificant bleeding when fragments of papilloma are teared off
2 The epithelial fragments are extremely well fixed and stained
3 Highly differentiated tumours of grades I and II which may be difficult to diagnose by urine cytology are easily identified
4 The extension of non exophytic lesions (carcinoma *in situ*) may be determined by means of multiple microbiopsies
5 Recurrences after radiotherapy may be verified earlier than by urine cytology as mitotic activity may be observed in fragments of malignant epithelium indicating a proliferative activity in tumour remnants

B Westermark, V Forsby, U Brunk, J Ericsson and J Ponten STUDIES ON IN VITRO CULTIVATED HUMAN GLIA AND GLIOMA CELLS, I AND II

Normal human glia cells (NGs) and glioma cells (MGs), the latter obtained from astrocytomas grade 3 and 4 (Kernohan), have been studied in tissue culture with special regard to cell movement and structure of the cell surface

I Cell movement has been studied by use of phase contrast microscopy and time lapse cinematography NGs showed well developed contact inhibition of ruffling of the leading edges and locomotion of the cells On the contrary in most MG lines where neither ruffling nor locomotion was inhibited following collision—resulting in crossing over the cells In some MG lines, cell locomotion ceased after contact, but ruffling remained uninhibited The ruffling of MGs was not restricted to the peripheral edge—as in NGs—, but often appeared on the upper surface ("atypical ruffling"), usually in the perinuclear zone

One to two days after infection of NGs with feline sarcoma virus, an abnormal pattern of ruffling and locomotion was observed with vigorous atypical ruffling on the upper surface of the transformed cells

The findings indicate that analysis of nuclear overlapping is not sufficient to detect lack of contact inhibition of ruffling The discrepancy between inhibition of ruffling and that of locomotion, as well as the finding of atypical ruffling emphasizes the need for time lapse cinematographical examination

II As demonstrated by transmission and scanning electron microscopy NGs are flattered with few microvilli and slender peripheral lamellae—corresponding to ruffling On the other hand MGs have numerous cytoplasmic extrusions with slender lamellae, not only at the periphery—like in NGs—but also on the upper surface—corresponding to

atypical ruffling Furthermore, MGs show extrusions which sometimes appear to have lost their cellular connection This vivid motility of the plasma membranes probably prevents formation of stable connections between MGs, such connections may be of importance for the regulation of cell proliferation

In NGs, endocytotic activity is most pronounced on the lower side, in contrast to the endocytotic activity encountered around the whole surface of the MGs which may reflect enhanced consumption of growth factors, possibly necessary for continuous proliferation

MGs contain much more numerous lysosomes and autophagic vacuoles than NGs Lysosomes are sometimes found in extrusions and detached cytoplasmic fragments The high autophagocytotic activity in the MGs could reflect increased turnover of plasma membranes and/or intracellular membranes

Our observations are compatible with two hypotheses for the invasiveness of malignant tumours

(1) lack of contact inhibition with constant cell movement, and
(2) "excretion" of lysosomal enzymes with digestion of intercellular substance

Acta path microbiol scand Section A 80, 697–704, 1972

INTERNAL ELASTIC LAMINA OF GASTRIC ARTERIES IN PSEUDOXANTHOMA ELASTICUM

Ultrastructural Studies

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Small arteries of the gastric wall of one patient with pseudoxanthoma elasticum (PXE) and four patients without this disease were examined For comparison skin of normal appearance from light protected areas of 5 PXE and 5 non PXE patients was studied In some of the gastric arteries of the PXE patient, the internal elastic lamina and surrounding tissues showed the typical ultrastructural alterations seen in PXE skin lesions i e large irregular calcium deposits inside and around elastic tissue, and in the surroundings a thready material of unknown nature and twisted, partly split collagen fibrils In addition calcification of collagen was observed Senile degeneration of the internal elastic lamina of gastric arteries and of dermal elastic fibres was observed in the controls as well as in the PXE patients No typical PXE change could be demonstrated in elastic tissue without histochemical evidence of calcification In PXE, calcification of the internal elastic lamina of gastric arteries seemed to precede a severe degeneration as well as a disruption of the internal elastic lamina and an irregular thickening of the intima

Pseudoxanthoma elasticum (PXE) is a genetically conditioned disease of connective tissue to appear in the skin, the eyes and the cardio vascular system The skin shows small yellow papules in the flexural folds the eyes angioid streaks in the ocular fundus and the cardio vascular system a widespread and early arteriosclerosis Ultrastructural studies of skin and endocardial lesions have previously been reported (*Loria et al* 1957, *Hashimoto & DiBella* 1967, *Huang et al* 1967, *Danielsen et al* 1970, *Pierard & Kint* 1970) Some authors (*Goodman et al* 1963, *Hashimoto & DiBella* 1967, *Danielsen et al* 1970) have reported a calcification of elastic fibres as the first visible abnormality in PXE Other authors (*Huang et al* 1967, *Pierard & Kint* 1970) have suggested that calcification takes place in an abnormal "granulofilamentous' material presumed to act as a precursor of elastic fibres The calcification was suggested to interfere with the development of the elastic fibre

The aim of the present study was to demonstrate

1 whether the ultrastructural alterations found in PXE skin lesions could be observed in gastric vessel walls as well,

2 whether PXE patients's gastric artery

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and dermal elastica with no histochemical evidence of calcification present any ultrastructural difference from those of non PXE patients,

3 whether a PXE calcification of gastric arteries precedes or follows other morphological changes in the vessel walls, particularly in their elastic tissue

MATERIAL

Small arteries from the gastric wall of one PXE patient aged 31 years and of 4 patients aged 40–60 years without PXE or any other systemic disease were examined All patients had a gastrectomy because of an ulcer of the stomach or duodenum Except for the gastrectomy, a clinical description of the PXE patient has been presented in a previous paper (*Danielsen et al* 1970, patient no 3) In addition skin of normal appearance from the gluteal region of five 20 to 65 year-old patients with PXE and from unexposed skin areas of five 20 to 63 year old normal subjects was studied An electron microscopic study of involved skin of the five PXE patients has been presented in the above mentioned paper of *Danielsen et al* (1970)

METHODS

For light microscopy paraffin sections were stained with Orcein for detection of elastic fibres and with Alizarin red S for demonstration of calcium salts

All gastric wall specimens were primarily formalin fixed and paraffin embedded For electron microscopy the paraffin blocks were deparaffinized in toluene overnight and transferred to a veronal acetate buffer pH 7 4 through a series of alcohols of decreasing concentrations The specimens were then fixed in a 1 per cent osmic acid solution in veronal acetate buffer pH 7 4 for one hour at 4° C Thereafter, the specimens were embedded in Epon 812 by the procedure generally followed. The skin biopsies removed from PXE patients were immediately fixed in a 6 per cent glutaraldehyde solution in veronal acetate buffer pH 7 4 with 7 5 per cent sucrose at 4° C overnight After washing



Fig 1 Normal internal elastic lamina containing patchy areas of calcified material (arrows) No elastic tissue alterations are seen around this material Gastric artery from a 31 year old PXE patient × 7,800



Fig 2 Area of Fig 1 in magnification × 80 000 Granules and thin needles (arrow) are noticed inside the dense material

the specimens were re fixed in a 1 per cent osmic acid solution in veronal acetate buffer pH 7 4 for one hour at 4° C, and dehydrated in alcohol of increasing concentration and embedded in Epon 812 For control of the method the skin biopsies from normal subjects were divided in two pieces and submitted to both procedures Ultrathin sections were cut by an LKB ultramicrotome and stained with uranyl acetate and lead citrate A Siemens electron microscope (Elmiskop IA) was operated at 80 kV with double condensors

RESULTS

Light microscopic studies Some of the arteries of the PXE patient showed calcification and rupture of parts of the internal elastic lamina as well as irregular thickening of the intima. Other arteries were of normal appearance The degree of calcification was increasing with the size of the vessels The arteries of the non PXE patients were normal or showed regular intimal thickening most pronounced in the larger arteries The skin of the PXE and the non PXE patients showed no abnormalities

Electron microscopic studies I The arteries of the PXE patient presenting light microscopic calcification of the internal elastic lamina showed large irregular areas of extremely electron dense material The electron dense areas showed granules and thin needles like calcium apatite crystals in random arrangement (Fig 2) The calcium deposits were seen either immediately below the endothelial cell layer (Fig 1) or separated from it by an irregularly thickened intima In the first mentioned areas, the lamina elastica interna appeared to be of normal shape and contained small patches of calcium, often completely surrounded by elastic fibre matrix (Fig 1), while in the other areas, the lamina was broken, split and con-



Fig 3 Broken internal elastic lamina containing large masses of calcified material (arrow) which also is seen in relation to collagen (c) or without relation to any of the two components (w) Many dense bodies (b) and areas of light granular material (m) are seen in the surroundings Gastric artery from a 31 year-old PXE patient × 7,800

tained larger and more continuous masses of calcium (Fig 3) In the broken parts of the lamina calcified material without relation to elastic tissue was also seen (Fig 3) In several sections of one of the arteries, some of the collagen fibrils around the broken parts of the internal elastic lamina were extremely electron dense containing thin needles remending of calcium apatite crystals In longitudinal sections, the dense material was strictly following the fibrils (Figs 3 and 4) It was presumed to be present in cross sections of the fibrils because many round, extremely electron dense cut surfaces with the size of collagen fibrils were observed The calcified fibrils were seen in close relationship to non-calcified fibrils showing distinct banding Many split and twisted collagen fibrils with increased diameters were observed in the calcified arteries Around the disrupted parts of the internal elastic lamina with extensive calcification, considerable masses of a thready material of unknown nature were also seen (Fig 5)

II Electron microscopy of the PXE patient s arteries in which histochemical evidence of calcification of the internal elastic lamina was absent, and of those of three of the non PXE patients did not reveal calcification of PXE type One of the control patients showed a tiny irregular area of dense material inside the internal elastic lamina of one of the largest arteries No PXE calcification was observed in skin of normal appearance from PXE patients The internal elastic laminae of gastric arteries and the dermal elastic fibres of all individuals showed various degrees of "senile degeneration in the form

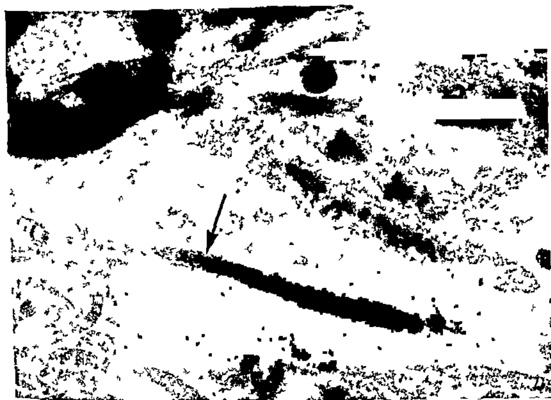


Fig 4 Dense collagen fibrils showing thin needles (arrow) Gastric artery of a 31 year-old PXE patient × 80 000

of (1) masses of dense grains (2) round holes in the matrix of the elastic fibres both being arranged in lines along the axis of the fibres The holes contained (a) granular material (b) thin banded rings (c) round electron dense bodies composed of granules or needles in annular or radial arrangement (Fig 7) (d) myelin like figures or (e) thin filaments with knobs probably representing acid glycosaminoglycans (*Kobayasi et al* 1971) Elastic fibrils were absent or very scarse inside the holes Slight moderate or severe senile degeneration of elastic tissue was found in the arteries of the non PXE patients (Fig 6) The luminar part of the internal elastic lamina was often most severely affected and the small vessels showed a more pronounced alteration of elastic tissue than the large The PXE patients gastric arteries without histochemical calcification showed senile degeneration of elastic tissue corresponding to findings in those of the non PXE patients The skin of normal appearance from the younger PXE and the younger non PXE patients showed slight degeneration of elastic tissue this degeneration was moderate in the normal skin from the older patients of both groups Twisting of collagen fibrils with increased diameters was observed in both PXE and non PXE arteries but in the skin only in one of the controls

III In the normally shaped areas of the internal elastic lamina the PXE patient s gastric arteries with histochemical calcification showed senile degeneration corresponding to findings in those of the non PXE patients and calcium deposits could be observed in completely normal areas of the lamina elastica interna (Fig 1) However the disrupted parts of the elastic lamina showed large amounts of degeneration products of elastic tissue in the surroundings of exten

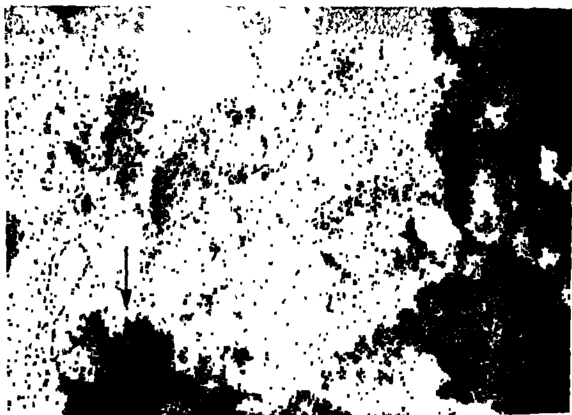


Fig 5 A thready material (t) around calcified areas (arrow). Gastric artery of a 31-year-old PXE patient × 60,000

sively calcified areas inside as well as outside the lamina (Fig 3)

There was no difference in the degeneration of elastic tissue in the paraffin-embedded and the non paraffin-embedded specimens

DISCUSSION

The authors realize that the material prepared for light microscopy is not sufficiently well preserved for electron microscopy However, fresh gastric wall from PXE patients to be used for study can rarely be obtained For this reason, the authors studied electron microscopically re embedded materials under careful control, *i e* PXE and normal gastric mucosa in re-embedded materials, and fresh and paraffin embedded normal skin As comparison of the two methods showed that formalin fixation of the material did not induce any change in dermal elastic tissue and as the electron microscopic finding of calcified material confirmed the light microscopic findings, we found the following interpretation of the results justified

The internal elastic lamina of some of the gastric arteries of the PXE patient showed ultrastructural alterations typical of PXE lesions of skin (*Danielsen et al* 1970) In contrast to the skin lesions, one of the arteries showed calcified material also inside or on collagen fibrils

The "senile" degeneration of elastic tissue observed both in gastric artery and skin was of the type previously found in dermal elastic fibres in relation to age and exposure to light (*Danielsen & Kobayasi* 1972) The finding of an equally pronounced degeneration in skin and arteries support the idea of an age related degeneration However, the finding of severe degeneration in the smallest

arteries and in the luminar part of the elastic lamina suggests that the degeneration of elastic tissue is dependent on factors other than age or exposure to light. The dense, round bodies represent a type of calcification related to "senile" degeneration of elastic fibres and should be distinguished from the PXE calcification and a calcification observed in arteriosclerosis in which the calcium deposits are situated in the matrix of the elastic fibre and show a random arrangement. The calcification in the wall of one artery of one of the non-PXE patients was of the same arteriosclerosis type (*Danielsen*, unpublished).

A preceding "senile-type" degeneration seemed not to be the cause of calcification of elastic tissue in PXE for the following reasons. "Senile" degeneration was found in elastic tissue of both PXE and non-PXE patients and, with the exception of the most extensively calcified areas, to the same extent. Moreover, calcification was seen in areas with no degeneration of elastic tissue as well as in areas with this degeneration. In addition, the "senile" degeneration appeared in lines parallel to the axis of the elastic fibre, while the calcification showed a random arrangement. The large amounts of degeneration products of elastic tissue observed only in the surroundings of extensively calcified and broken areas of the internal elastic lamina may well be secondary to the calcification. Our findings cannot support the theory advocated by *Huang et al.* (1967) according to which calcification may take place primarily in the perielastic tissue, as we found calcified areas surrounded by matrix inside the elastic fibre. The presence of calcification also outside elastic tissue may be due to a continuous growth of the calcified plaques together with a degeneration of the elastic tissue and a spreading of the degeneration products to the



6. Internal elastic lamina with masses of dense grains (g) and many holes (h). Gastric artery of a 40-year-old non-PXE patient. × 7,800



Fig 7 Dense round body showing needles (arrow) in radial and annular arrangements Gastric artery of a 40 year-old non PXE patient × 80 000

surroundings Neither did we find support for the suggestion voiced by *Huang et al* (1967) and *Pierard* & *Kint* (1970) that an abnormal "granulofilamentous' material (identical with our thready material) could be the origin of the elastic tissue calcification in PXE, as this material only was observed in areas with extensive calcification The irregular intimal thickening seemed to occur secondarily to the calcification in the PXE patient There is no answer to the question, why morphologically normal elastic fibres of PXE patients calcify

The authors wish to thank *Svend Petri*, M.D and *Gunnar Teilum*, M D, who placed the gastric mucosae at our disposal

We also wish to thank Miss *Lise Fredebo*, Mrs *Nancy Hansen* and Mr *John Winther* for their valuable technical assistance

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LIGHT MICROSCOPICAL FEATURES IN LIVER BIOPSIES WITH MALLORY BODIES

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A s udy of the morphology and s a n ng reac ons of Mallory bod es s g en on the bas s of a consecu e se es of 62 l ver b ops es w h Mallory bod es Two types of Mallory bod es are desc bed acco d ng to s ze and age The d ffe ent al d agnos c problems are d scussed and the h s olog cal changes n l ver b ops es conta n ng Mal ory bod es a e de cr bed

In 1911 F B *Mallory* (12) described a peculiar cytoplasm c l yal ne structure in l er cells of alcohol cs with c rrhos s and s ea osis He cons dered the les on to be a spec f c f nd ng in alcohol cs and named it alcohol c hyal ne

In later works (1 20) alcohol c hyal ne or Mallory bod es have been demonstrated n c rrhosis both w th and witl out fatty infl trat on and in fatty l ver w th no cirrhos s (16)

Over the years intracytoplasm c inclus ons were observed n l ver w tl out c rrhosis or fatty change (14 21 22) as well as n l vers of exper mental an mals (17) These inclu s ons were often compared to and n certa n instances ere fo nd o be dent ca Mal lory bod es but vere in other cases both in humans (14 22) and in exper mental an mals (17) m s nterpretated as Mallory bo d es

Partly because of the above mentioned m s n erpretat ons and partly because most ma erials deal ng w th the morphology of Mallory bod es are selected non-consecut ve series (1 20) tl ere is in the l terature some uncertainty both w th regard to the morpho logy of Mallory bod es and w th regard to the k nd of changes encountered n l ver t ssue conta n ng Mallory bod es

The ma n object of th s work has been to describe the morphology of Mallory bod es and the cytolog cal and h stolog cal changes in l vers w tl Mallory bodies on the bas s of a consecutive ser es of liver b ops es

It is further noted by several authors (8 10 12) that Mallory bod es frequently are diff cult or mposs ble to ident fy in haemato xylin eos n preparat ons For tl ese reasons spec al methods of sta n ng have been re commended 2 8 0 19)

Another object of the work presented l as therefore been to invest gate if there are any d fferences n tl e morphology and the num bers of Mallory bod es demonstrated w th d fferent sta n ng methods

MATERIAL AND METHODS

The ma erial cons s s of a o al of 62 pe cutane ous l er b ops es all exh b ng Mallory bod es The ma erial has been se ec ed as consecut e

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biopsies showing Mallory bodies from a total of 1,100 percutaneous liver biopsies (5) received at the Pathological Anatomical Institute at the Kommunehospitalet during the period October 1st 1965 to October 20th 1967

The tissue has been fixed in neutral formalin and imbedded in paraffin Ten to fifteen sections were cut on a sledge microtome from the first 23 biopsies while about 40 serial sections were cut on a rotary microtome from the remaining 39 biopsies The sections are approximately 6 μm in thickness

Morphology The part of the present work which aim was to examine the morphology of Mallory bodies and the cytological and histological changes in livers with Mallory bodies has been performed on haematoxylin and eosin and v Gieson Hansen stained sections Additional sections stained for iron (15) reticulin (9) and pyroninophil substance (4) have been available from all biopsies

The localization of Mallory bodies in the parenchyma was estimated and Mallory bodies have been quantified in the following manner

- \+ on an average not more than one Mallory body per mm tissue
- \+ + on an average more than one Mallory body per mm tissue but less than five
- \+ + + on an average more than five Mallory bodies per mm tissue

In addition to an assessment of the number of Mallory bodies (+ + +, + + +) a series of morphological changes mentioned in Table 2 have been semiquantitatively registered

Furthermore it has been registered whether cirrhosis is present (defined as liver tissue with nodular regeneration and fibrosis) as well as the degree of fibrosis and the content of iron in liver cells and Kupffer cells (0–3) + indicates slight + + moderate and + + + severe changes

Staining characteristics In 34 of these 62 biopsies the number of Mallory bodies in each section was so high and the amount of tissue remaining in the paraffin block after routine histological sections had been cut sufficient to warrant a study of the staining characteristics of Mallory bodies to be carried out

After sections for routine histology had been cut serial sections for the staining procedures listed in Table 1 were cut Every second section was stained with routine haematoxylin and eosin

In order to investigate if it was possible to demonstrate Mallory bodies with the above mentioned staining procedures in liver biopsies with no Mallory bodies in haematoxylin eosin stained sections the same staining procedures had been applied to another group of 75 percutaneous liver biopsies This group had been selected as consecutive biopsies with cirrhosis and fatty change but no Mallory bodies from the same 1 100 biopsies mentioned above (6)

In seven cases it has been possible to supplement the investigation with frozen sections of unfixed tissue followed by phase contrast microscopy of the unstained sections and in six cases Epon imbedded material was at hand for examination of the sections after toluidine blue staining

RESULTS

A number of morphological characteristics relating to Mallory bodies will first be presented, and then the frequency of these and other changes in liver tissue containing Mallory bodies will be given

Morphology of Mallory bodies The structure registered as Mallory bodies in this paper presents one of the following appearences, though it must be noted that there are gradual transitions between the types

Type 1 spherical, homogeneous, and eosinophilic cytoplasmic masses with a diameter 2 3 μm They are less intensely stained than type 2 described below just as the demarcation towards the surrounding cytoplasm is less well defined These spherical structures are sometimes found isolated or in small groups of two to three elements which still are distinctly separate (Fig 1), but are most frequently found partly confluent forming twisted structures (Fig 2) or irregular reticular masses where the individual elements still may be identified at high magnification

Type 2 irregular highly eosinophilic masses of hyaline which sometimes nearly fill up the cell The large masses often have a pale stained center surrounded by a bright rim of hyaline (Fig 3)

Sometimes the same cell may contain Mallory bodies of both type 1 and 2 or a Mallory body is found which to one side is type 1 and to the other side is type 2 (Fig 4)

Staining characteristics of Mallory bodies The appearance of Mallory bodies when stained with haematoxylin and eosin has been described above A summary of the methods



Fig 1 Enlarged liver cells with a vacuolated empty looking cytoplasm The arrows indicate a liver cell with an ill defined cell boundary and a central group consisting of three elements of Mallory bodies type 1 To the left of the cell neutrophils are seen H E × 1000



Fig 2 Enlarged vacuolated liver cell surrounded by neutrophils In the empty looking cytoplasm a twisted mass consisting of partly confluent Mallory bodies type 1 is seen The liver cell is surrounded by neutrophils

employed and the results obtained is given in Table 1

In unstained sections examined by phase contrast microscopy Mallory bodies were visible as intracytoplasmic refractile networks with an appearance similar to that seen in stained sections

With Luxol fast blue Mallory bodies appear as well defined blue masses and with chromotrope aniline blue as blue structures With acid fuchsin containing stains Mallory bodies retained the acid fuchsin during decolorization and appeared as bright red aggregates

After methylene blue staining the Mallory bodies were readily identified as prominent red structures in the liver cells and with phosphotungstic acid haematoxylin the Mallory bodies appear as a purple perinuclear cellular component

In thin Epon imbedded sections stained with toluidine blue the configuration of Mallory bodies was polymorphic with confluent perinuclear clumps displaying various shades of blue

No Mallory bodies were demonstrated by the employed staining procedures which could not be identified in the neighbouring haematoxylin eosin stained section Nor were any Mallory bodies demonstrated in any of

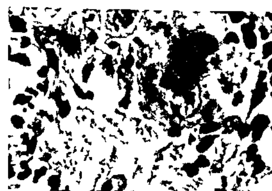


Fig 3 Liver cell containing a large Mallory body type 2 in its upper part and in its lower part a crescent shaped mass consisting of partly confluent Mallory bodies type 1 H E × 1000



Fig 4 Liver cell containing Mallory bodies which in its upper part correspond to type 2 while they in its lower part consist of several small elements of type 1

TABLE 1 *Staining Reactions for the Demonstration of Mallory Bodies*

Reaction	Colour of Mallory bodies
Luxol fast blue	purplish blue
Chromotrope aniline blue	blue
Phloxine methylene blue	intense red
Phosphotungestic acid	purple
Acid fuchsin	red
Toludine blue	blue

the sections from 75 biopsies constituting the control series

Changes in and around liver cells containing Mallory bodies Liver cells containing Mallory bodies are often enlarged and most frequently vacuolized with faintly stained cytoplasm but without lipid inclusions In many of the affected cells the nucleus had a hyperchromatic appearance, and sometimes cells containing Mallory bodies are shrunken with indistinct nucleus and the hyaline is quite dense In *alcoholic hepatitis* a few or several liver cells containing Mallory bodies are seen exhibiting changes as in necrosis in the form of indistinct cell boundaires, karyorrhexis, and infiltration of the vicinity with neutrophils The changes described in alcoholic hepatitis may affect solitary liver cells containing Mallory bodies a single cell in a larger group of cells with Mallory bodies, or all the cells in the group (Fig 5) The use of serial sections reveals that neutrophils

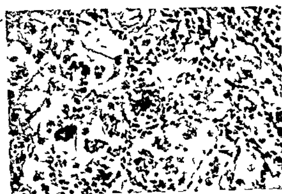


Fig 5 Group of liver cells with Mallory bodies The cell borders are indistinct and the cells are surrounded by neutrophils H E × 224



Fig 6 Centrilobular area with alcoholic hepatitis and beginning collagen formation especially to the right of the central vein H E × 250

in the parenchyma nearly always lie in relation to liver cells containing Mallory bodies

Sclerosing hyaline necrosis is a form of alcoholic hepatitis in which the central veins become involved in centrilobular scar tissue (7) Initially the histologic features of alcoholic hepatitis are present The collagen proliferation within the central sinusoids tends to obliterate the spaces formerly occupied by parenchymal cells At first this is a lattice like network (Fig 6) Later the connective tissue condenses and becomes a confluent mass that produces obliteration of sinusoids central veins, and sublobular veins The veins are difficult to identify in these scars

Topographical features Liver cells with Mallory bodies occur focally, singly, or in small groups and appear in all areas of the lobule or regenerative noduli, but most frequently in relation to the central vein in liver tissue with preserved architecture and in the marginal areas of the noduli in liver tissue with destroyed architecture The number of liver cells with Mallory bodies varies from one solitary per biopsy to numerous per high power field of vision

In liver biopsies with Mallory bodies greater or smaller areas, most often but not consistently in relation to liver cells with Mallory bodies, are found, where the liver cells assume a balloonlike appearance in which the nucleus is the only organized structure recognized (Fig 1)

TABLE 2 Number of Biopsies with the Following Parenchymal Changes

Parenchymal changes		
Mallory bodies	+	41
	+ +	16
	+ + +	5
Alcoholic hepatitis		45
Sclerosing hyaline necrosis		8
Fatty change	+	21
	+ +	35
	+ + +	6
Lipogranulomas		47
Focal necroses		57
Acidophilic bodies		50
Kupffer cell proliferation		62
Parenchymal inflammation		56
Cholestasis		9

TABLE 3 Number of Biopsies with the Following Changes in Connective Tissue

Connective tissue changes	
Inflammation	62
Bile duct proliferation	44
Fibroses	60

Quantitative study of morphological features

A summary of the parenchymal changes is given in Table 2

Mallory bodies In 41 of the 62 biopsies a small number of Mallory bodies is demonstrated while 16 exhibit a moderate, and five a great number

Alcoholic hepatitis 45 of the 62 biopsies (73 per cent) exhibit alcoholic hepatitis In 34 the changes are slight while 11 exhibit moderate to severe alcoholic hepatitis

Sclerosing hyaline necrosis' in slight degree was seen in five and in severe degree in three of the 17 biopsies without cirrhosis

Fatty change and lipogranulomas There is a slight fatty infiltration in 21 biopsies moderate in 35 and severe in six Lipogranulomas are seen in 47 (76 per cent) of the 62 biopsies

Focal necroses are observed in the majority of biopsies (57 (92 per cent)) In none of the cases were confluent or many focal necroses found

Acidophilic bodies are found in 50 biopsies The number of acidophilic bodies is in all cases small

Mesenchymal reaction in the parenchyma In 54 cases there is a slight and in eight cases a moderate Kupffer cell proliferation

The degree of inflammation in all 56 biopsies with inflammatory cells is slight Lymfocytes are encountered in 42 biopsies, while neutrophil granulocytes are found in 47 cases The neutrophil granulocytes are in 45 of these 47 biopsies predominantly found in relation to necrotic liver cells with Mallory bodies i e as an integrating component of the alcoholic hepatitis Plasma cells have only been demonstrated in a total of three biopsies and eosinophilic granulocytes in a total of two

Cholestasis Only a minority of the biopsies contain bile thrombi and the cholestasis is slight in all nine cases

Iron and lipofuscin content The majority of biopsies contain no iron either in liver cells or in Kupffer cells while lipofuscin is seen in all 62 biopsies, in a slight degree in 21 and a moderate degree in 41

A summary of the *changes in the connective tissue* is given in Table 3

Inflammation All biopsies show portal inflammation Lymphocytes are demonstrated in all biopsies macrophages in 57 and neutrophils in 28 biopsies The number of biopsies with plasma cells is 39 with eosinophils eight

Bile duct proliferation Bile duct proliferation is seen in the majority of biopsies and is of a slight degree in 13 biopsies and of a moderate to severe degree in 31 biopsies

Fibroses There is fibrosis in all biopsies save two and in the vast majority of cases this is a moderate to severe fibrosis

Cirrhosis This is encountered in 45 of the 62 biopsies 26 of these 45 biopsies with cirrhosis exhibit a total destruction of the normal lobular architecture while 19 still show some remnants of the original tecture

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Acta path microbiol scand Section A 80, 713 717, 1972

HETEROTRANSPLANTATION OF HUMAN EPIDERMOID CARCINOMAS TO THE MOUSE MUTANT *NUDE*

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Successful heterotransplantation is reported for 3 out of 4 human epidermoid carcinomas consecutively transplanted to the mouse mutant *nude* Serial transmission was obtained in two cases so far for 2 5 passages Tumours grew locally in mice and no metastases were observed Histological and cytological appearance of the grafts were in full accord with the original human tumour The mouse mutant *nude* is suggested as a host for human epidermoid carcinomas in the study of oncostatic agents

The need for simple, *in vivo* models for the study of the action of anti cancer agents on human cancers is urgent The mouse mutant *nude* which is immunologically incompetent due to recessive thymic aplasia, may be of use in such studies We have previously successfully transplanted human adenocarcinomas of the colon (4, 6) and human malignant melanomas (3) to this mouse mutant

The purpose of this paper is a study of the growth pattern of 4 human epidermoid carcinomas in this mouse mutant Heterotransplantation of this group of tumours is of special interest because the action of oncostatic agents (*e g* bleomycin (1 8)) known from clinical practice to be effective on such carcinomas may perhaps be tested on this model

MATERIALS AND METHODS

Mice Six–eight weeks old *nude* mice of both sexes bred at Pathological Anatomical Institute Kommunehospitalet Copenhagen according to the principles previously described (5) were inoculated Following inoculation of tumour tissue the animals were observed daily and all were autopsied

Tumour tissue Three of the tumours in this series were from patients treated consecutively at Kommunehospitalet Copenhagen within a period of one month and were transferred directly from patients to mice Biopsies from the fourth tumour originating from a patient at Kenyata National Hospital, Nairobi Kenya were obtained in collaboration with Professor *George Klein* and Dr *Eva Klein* Karolinska Institutet Stockholm Sweden During aerial transport (Nairobi Stockholm–Copenhagen) these biopsies were kept in specimen holders with a culture medium (Eagle s minimal essential medium with 10 per cent fetal calf serum) in a container with crushed ice

Method of inoculation In all cases solid blocks of tumour tissue measuring 2×3×3 mm were inoculated subcutaneously in the lateral abdominal wall as previously described (6) At the primary inoculations 2 5 *nude* mice were used The same method of inoculation was used in the later serial transplantations into 1 10 *nude* mice according to the amount of tumour available

As anaesthetics we used ether or an intraperitoneal anaesthetic with propanididum (Epontol® 0 5 mg g mouse)

Clinical data dates of inoculations origin of the tumour inocula and time from removal of the tumour until inoculation are seen from Table 1

Specimens for histologic examination have been

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TABLE 1 *Clinical Data Dates of Inoculations Origin of Tumour Inocula, Time before Inoculations and Results of Transplantations*

Experiment no	Data of patients	Primary human tumour	Previous treatment	Date of inoculation	Origin of tumour inocula	Time before inoculation	Take	Number of transfers	Rate of take in the passages
1	65 ♂ KH 1075/70	Bronchogenic epidermoid carcinoma with cornification	0	11 2 1970	Cutaneous metastasis	20 min	+	2	2/4 1/4
2	63 ♂ KH 1372/70	Laryngeal epidermoid carcinoma with cornification	High voltage therapy (Stabilatron) July–Aug 1969 Bleomycin (Total dosage 75 mg i v) Jan Feb 1970	26 2 1970	Primary tumour	Approx 60 min	0	0	0/2
3	72 ♂ KH 1625/70	Maxillary epidermoid carcinoma with cornification	High voltage therapy (Stabilatron) Oct Dec 1969	9 3 1970	Primary tumour	Approx 45 min	+	1	1/5
4	55 ♂ IP 10679/71	Maxillary epidermoid carcinoma with cornification	0	18 8 1971	Primary tumour	Approx 48 hours	+	5*	3/4 12/21 9/19 8/27 19/29

* March 1972

fixed in formalin imbedded in paraffin sectioned at 7 μm and stained with H & E van Gieson Hansen and the methyl green pyronin method (2)

RESULTS

As it is seen from Table 1 growth of the grafts was obtained in 3 out of 4 cases (Exp No 1, 3 and 4) Serial transplantation of the tumours succeeded in 2 of these cases (Exp No 2 and 4) in 2 and so far, 5 passages respectively The rate of takes in the different passages is also given in Table 1

General observations The general condition of the tumour grafted mice does not seem to be influenced and the life span of the animals was unchanged They became 4–5 months old, and died from wasting, like untreated animals

Mode of growth and macroscopic appearance of the tumours in the mice In 2 cases (Exp No 1 and 3) recognizable tumour growth was seen at the site of inoculation after 10–14 days These tumours grew slowly, never surpassing 5×5×5 mm at the time of the animals' death

In the third positive case (Exp No 4) recognizable tumour growth appeared at about the same time The tumours in this case showed considerable growth energy, growing steadily until the death of the animals up to a size of 15×15×20 mm at the most This growth pattern was constant through serial passages

In all cases the tumours showed only local growth without metastasis to lymphnodes or organs

The tumours were well circumscribed, situated in the subcutaneous space the smaller showing (Exp No 1 and 3) a homogenous greyish cut surface without recognizable necrosis The tumours in Exp No 4 showed a pronounced tendency to central necrosis resulting in a cystic appearance with a peripheral zone of viable tumour tissue of variable breadth The tumours are supplied from the region s superficial arteries and veins which both dilate and ramify strongly concurrently with the growth of the tumour



Fig 1 Low power view of 2nd passage Experiment No 1, 56 days after inoculation The tumour is well circumscribed lying in the subcutaneous space between the skin and the muscular layers of the abdominal wall 40 × H & E

Microscopic appearance The investigated tumours were all well circumscribed surrounded by a condensed layer of connective tissue, containing numerous large vessels and microscopic examination showed them restricted to the subcutaneous space (Fig 1) Larger tumours were accompanied by some flattening of the muscles in the skin and abdominal wall The histologic and cytologic appearance of the tumours corresponds closely to the human inoculum (Figs 2 and 3) Invasion of tumour tissue into lymphatics or blood vessels was not demonstrated

In the following a short histologic description is given of 1) sections of human donor material taken close to the tumour inoculum and of 2) the tumours as appearing in the recipients When the former were insufficient for a final diagnosis, supplementary sections were taken from other parts of the tumour

Experiment No 1

Donor material Cutaneous metastasis from well differentiated epidermoid carcinoma with cornification A few small necroses are seen Many mitoses, of which several show abnormal configuration The stroma is sparse with minimal inflammatory reaction consisting in a few lymphocytes

Recipients (1st and 2nd passages) Histologic and cytologic appearance as donor material

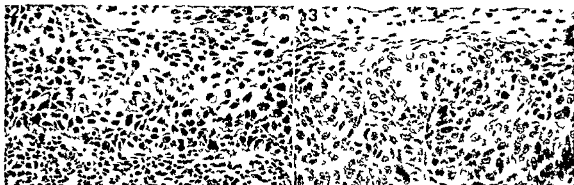


Fig 2 Section from human donor material of Experiment No 4 Well differentiated epidermoid carcinoma 250 × H & E

Fig 3 Experiment No 4 3rd passage in *nude* mice The histological and cytological picture is the same as seen in Fig 2 250 × H & E

Experiment No 2

Donor material Fragmented connective tissue stroma with widespread necroses and only few areas of morphologically preserved epidermoid carcinoma Additional sections from more remote areas of the tumour show well differentiated epidermoid carcinoma with cornification

Recipients No take

Experiment No 3

Donor material Fragmented fibrous stroma with slender streaks of epidermoid carcinoma Sections from more remote parts of the tumour material showed well differentiated epidermoid carcinoma with cornification

Recipients (1st passage) The material is very sparse a few small pieces of an epidermoid carcinoma with the same degree of differentiation as in the human donor material Mitoses of which a few exhibit atypical configuration are seen in the tumour tissue The stroma plentiful with slight cellular infiltration predominantly lymphocytes

Experiment No 4

Donor material Fragmented tissue partly covered by respiratory epithelium consisting of smaller and greater partly confluent areas of a well differentiated epidermoid carcinoma with extensive parakeratosis and cornification Many mitoses some of atypical configuration Only slight central necrosis Stroma scant with heavy infiltration of neutrophils and round cells (Fig 2)

Recipients (1st–5th passages) Well differentiated epidermoid carcinoma with central parakeratosis and cornification Many mitoses several with abnormal configuration The extent of the necroses increases with the size of the tumours so that the larger tumours exhibit confluent central necroses and considerable amounts of horny material The amount of stroma is variable most often scant with very slight infiltration with round cells (Fig 3)

DISCUSSION

The experiments described have shown that it is possible to transfer human epidermoid carcinomas to *nude* mice although with considerable variation in takes and rate of growth The mode of growth of the tumours has in other respects been the same and the histologic and cytologic appearance of the tumours in the mice was in all cases in accordance with the human donor material

In 3 out of 4 attempts growth of tumour was obtained in the recipient mice In 2 cases (Exp no 1 and 4) serial transplantation was successful in 2 and at the moment 5 passages, respectively In these cases the tumour tissue has been obtained from tumours

previously untreated In one case growth of the tumour tissue was only obtained in the first passage, and here only in 1 out of 5 animals (Exp No 3) The reason for the relatively bad result in this attempt at trans plantation may be previously given X ray therapy This is supported by the negative outcome of the transplantation in Experiment No 2 This donor had likewise previously been given X ray therapy for his tu mour and had furthermore received bleomycin 14 days before the attempt at transplantation

The rate of growth varies considerably be tween the tumours in this series, in spite of a fairly uniform degree of differentiation as assessed by ordinary histologic criteria

In Experiment No 1 and 3 the tumours exhibit a low degree of growth energy in mice, while the tumours in Experiment No 4 grew rapidly and with constant rate of growth through the series

The rate of growth is essential as it must be seen in relation to the short life span of *nude* mice under the described conditions (approx 4–5 months *i e* approx 2–3 months after inoculation) To be applicable in this system a tumour must within this period of time attain a size in the animal, allowing transfer to several others The use of *nude* mice kept in germfree conditions which may be expected to live up to 18 months (7), will make it possible to work also with slowly growing tumours in this model

In all cases the tumours grow as local processes at the site of inoculation without tendency to metastasize to lymphnodes or organs like in previous attempts at heterotransplantation of other kinds of human malignant tumours to *nude* mice (3, 4, 6)

The histologic and cytologic appearance of the tumours in the mice corresponds exactly to the original apart from lacking the capability of infiltrative growth No changes in the microscopic appearance have been observed through the series Also this finding is in accordance with observations on trans plantation of other human malignant tumours to *nude* mice (3, 4, 6) Chromosome analysis of some of these tumours showed in all cases a human chromosome pattern (9)

The time from removal of the tumour tissue until inoculation in the animals varied between 20 minutes and 48 hours, which seems insignificant as the best result was obtained after a period of 48 hours

The model described here may be considered in the light of clinical results with bleomycin, which has an oncostatic effect on human neoplastic diseases, especially epidermoid carcinoma (1, 8)

Our thanks are due to the Otorhinolaryngologi cal Department and Surgical Department I, Kommunehospitalet, Copenhagen as well as to Profes sor *George Klein* and Dr *Eva Klein*, Karolinska Institutet Stockholm for the tumour material

The work has been carried out with grants from the *Dagny and Harry West Jørgensen's Foundation* and *The Danish Anti Cancer League*

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Acta path microbiol scand Section A *80*, 718–720, 1972

OCCURRENCE OF AMYLOID DEPOSITS IN THE SKIN IN SECONDARY SYSTEMIC AMYLOIDOSIS

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Among 9 patients with secondary systemic amyloidosis, 8 had amyloid deposits in the skin Amyloid was found in the deepest part of the dermis around the adnexa and in the subcutaneous tissue in the form of rings around fat cells The amount of amyloid varied somewhat between different skin areas and, among the parts of the body examined, the *scalp and the abdominal skin showed the heaviest infiltration* It is obvious that secondary amyloidosis can be diagnosed by means of skin biopsy in many cases

In secondary systemic amyloidosis, amyloid is deposited mainly in certain parenchymatous organs such as the kidneys, adrenals and spleen Amyloid infiltration also affects blood vessels in the gastro-intestinal tract, among other organs which is the reason why rectal biopsy is the most commonly employed diagnostic method It has recently been found (4) that amyloid deposits may be found in the abdominal skin The present report describes the occurrence of amyloid in the skin of different parts of the body in secondary systemic amyloidosis associated with rheumatoid arthritis

MATERIAL AND METHODS

During a period of 9 months 726 adult patients were autopsied at the Institute of Pathology in Uppsala Among these 25 (3 4 per cent) had a clinical diagnosis of rheumatoid arthritis From these patients tissue specimens were taken from the liver spleen kidney and rectum Skin specimens including subcutaneous tissue were taken from the following areas 1) Immediately behind the ear, 2) The lateral area of the upper arm 3) The abdomen at the midline between the *umbilicus and the xiphoid process*, 4) The ventral side of the thigh, and 5) The plantar side of the great toe The tissue specimens were fixed for 24 hours in 10 per cent formalin and 10 per cent buffered neutral formalin embedded in paraffin and sectioned The sections were then stained *with van Gieson stain* and alkaline Congo Red (2) and were studied in polarized light The amount of amyloid in the different sections from the kidney rectum and skin was graded from + to + + + Two cases of rheumatoid arthritis with known amyloidosis have since been added to this material

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RESULTS

Seven (28 per cent) out of the 25 patients with rheumatoid arthritis showed deposits of amyloid in the parenchymatous organs, and the same applies to the two patients with known amyloidosis In eight of the patients, amyloid deposits were observed in vessels of the submucosa and mucosa of the rectum (Table 1) Deposits of amyloid in the skin were seen in all of these eight cases The deposits were always limited to the deepest part of the dermis and the subcutaneous tissue In the dermis, the infiltration was observed as thin amyloid rings around sweat glands, seb

TABLE 1 *The Amount of Amyloid in the Investigated Specimens from 9 Patients with Secondary Amyloidosis*

Case	Kidney	Rectum	Skin Head	Skin Arm	Skin Abdomen	Skin Thigh	Skin Toe
1	+	+ +	0	0	+ + +	0	0
2	+ + +	+ + +	+	0	+	0	0
3	+	+	+	+	+	—	—
4	+ +	+ +	+	+ +	+ +	+	+
5	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
6	+ + +	—	—	—	—	—	—
7	+ + +	+ + +	+ + +	+	+ + +	+ +	+ +
8	+ +	+ +	+ +	+	+ +	+	—

0 – not investigated
— to + + + different amounts of amyloid The quantitation of amyloid in the skin refers to the subcutaneous layer only

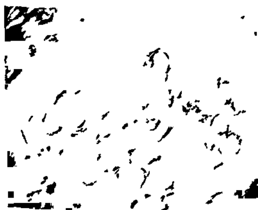


Fig 1 Amyloid deposits around sweat glands Congo red stain with polarized light × 150



Fig 2 Sweat glands and fat cells (arrow) with amyloid deposits Congo red stain with polarized light × 270

aceous glands and hair follicles (Figs 1 and 2) The deposits were present in patches and completely unaffected adnexa were often found close to adnexa with amyloid deposition In the subcutaneous tissue deposits of amyloid occurred as rings around fat cells (Fig 2) Regions with such fat cell alterations varied greatly in size but practically all fat cells were surrounded by amyloid in some cases Small blood vessels infiltrated by amyloid were seen both in the subcutaneous tissue and in the deep layers of the dermis The amount of amyloid in the different skin regions in different patients varied somewhat (Table 1) There was good agreement between the quantities of amyloid in the kidney and rectum but agreement between those in the kidney and skin was poorer In one patient (case no 6), abundant amyloid deposits were found in the kidney but none in the rectum or skin This patient showed only mild vascular amyloidosis in the liver and spleen

DISCUSSION

The occurrence of amyloid in the skin in secondary amyloidosis was reported at an early date (3) but has since been doubted (1 In a previous study, however (4) I found amyloid deposits in abdominal skin in four out of five examined cases with syste

mic secondary amyloidosis Among the nine patients seen in the present study in whom secondary amyloidosis was associated with rheumatoid arthritis, eight exhibited amyloid infiltration in the skin The deposits were always localized to the deepest part of the dermis and the subcutaneous tissue and never formed any major coherent mass On the other hand, in primary systemic amyloidosis, amyloid is often found in the papillary plexus of the dermis and even diffusely throughout the dermis (1) The occurrence of amyloid rings around fat cells in the subcutaneous tissue is, however, common to the two forms of amyloidosis

The amount of amyloid seems to be somewhat greater in the scalp and abdominal skin than in the other skin areas examined The abdominal skin is easily accessible for biopsy and it has been shown recently (5) that the diagnosis of secondary amyloidosis can be made by means of fine needle biopsy of the subcutaneous tissue in the area between the umbilicus and the xiphoid process

Skin biopsy can thus be used for the diagnosis of secondary systemic amyloidosis It does not seem possible, however, to draw any definite conclusion from a skin biopsy concerning the amount of amyloid in other organs, *e g* the kidneys

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Thanks are due to *Ulla Hansson, Ann Charlotte Lundstrom, Lena Ronning* and *Anders Strand* for skilled technical assistance The study was supported by a grant from *Konung Gustaf V s 80 års fond'*

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Acta path microbiol scand Section A 80, 721-728, 1972

QUANTITATIVE STUDIES OF THE RENAL CORPUSCLES II: A METHODOLOGICAL STUDY

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The camera lucida drawing method with planimetric area measurements was compared with direct microscopy and systematic point count in a quantitative study of glomeruli the study including differential counts of nuclei and determination of the total and mesangial areas Good reproducibility was found using both methods, but the camera lucida drawing method was found to be at least three times more time consuming than direct microscopy for total and differential counts of nuclei and systematic point count for mesangial area determination Measurement of total glomerular area was done planimetrically, by point counting and by determination of the area as an ellipse or a circle The results obtained planimetrically and by point counting did not differ significantly, whereas the difference between the results of the planimeter measurements and the results obtained by applying the formula of an ellipse or a circle on glomerular sections was found to be statistically significant If central sections were compared with random sections of glomeruli, deviation of results was found to be greater in the random sections, and it was calculated that 10 central and about 30 random sections have approximately the same value in quantitative examination of a renal tissue specimen Thus, it appears advisable to use direct microscopy for total and differential counts of nuclei, systematic point count for mesangial area determination, planimetric measurement or point counting for total glomerular area determination and to apply this method on central sections of glomeruli

In quantitative studies of human glomeruli two methods have been used for the determination of mesangial area and differential counts of nuclei *Kimmelstiel* and co-workers (*Iidaka et al* 1968, *Kawano et al* 1969, *Kawano et al* 1971) and *Hanberg Sorensen* (1972) have employed camera lucida drawings combined with planimetric area measurements, whereas *Wehner*'s group has preferred direct microscopy for differential counts of nuclei and a point count method for determination of mesangial area (*Wehner & Anders* 1970, *Wehner et al* 1970)

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Previous studies of intraglomerular variation have revealed the existence of a central area where values of the examined parameters are relatively constant For this reason Kimmelstiel and co workers and Hanberg Sorensen have used only sections from the central half of the glomeruli in their studies Wehner on the other hand, used all glomerular sections regardless of their relationship to the first and last section in the series of sections (random sections) This has been done from the point of view that the most correct information of glomerular changes is obtained when the first, the central and the

last glomerular sections are represented (*Wehner* 1971)

In evaluations of renal biopsies by conventional microscopy, a question arises as to the numerical representation of glomeruli in a specimen required in order that the latter may be considered acceptable This problem has previously been thoroughly investigated by *Thomsen* (1965) Theoretically, quantitative study of glomeruli should be performed in cases where conventional microscopy has revealed questionable glomerular changes However, in order to do a quantitative examination in such cases, the relevant para meters must be adequately described on a number of glomeruli that is usually seen in a renal biopsy

The purpose of the present study was 1) to determine which one of the above mentioned methods, or some combination of ele ments from both methods, should be used in quantitative studies of glomeruli and 2) to investigate the precision by which the examined parameters were determined in ten glomeruli using both central sections and random sections Ten glomeruli were chosen as this number is present in most biopsies

MATERIAL AND METHODS

The normal renal tissue studied originated from a 47 year old woman with no history of renal disease who had died of a ruptured intracranial aneurysm Both kidneys were removed immediately after death The right kidney was used as a renal transplant while the left kidney was used in the study reported here and in the pervious study of intraglomerular interglomerular and interfocal va riations in the normal kidney (*Hanberg Sorensen* 1972)

Tissue specimens were fixed in Carnoy s fluid for 30 hours with the exception of one specimen which was fixed in Helly s fluid for one hour This latter specimen was employed in an auto blind study of the camera lucida drawing method The reason why Helly s fluid was used instead of Carnoy s fluid was that this fixative apparently produced less fainting of the mesangium and the basement membrane after prolonged exposure to bright light The paraffin sections were cut with a microtome adjusted to 2 μm and stained with periodic acid Schiff haematoxylin A Leitz Ortolux microscope with a mirror for projection was used for all examinations The objective used for camera lucida drawing was a Leitz Pl Apo Oel 100/1,32 while the objective used for direct microscopy and point counting was a Leitz A 45/0,65

Using the camera lucida drawing method, the total linear magnification was × 1350 (oil immer sion) With this technique the glomeruli were projected onto the table in front of the microscope and drawn on paper The drawing included the in ner limit of the Bowman's capsule the basement membrane of the capillaries, the mesangium and the glomerular nuclei which were identified as mesangial, endothelial and visceral epithelial nuclei according to their position in relation to the mesangium and the basement membrane In cases of doubt, nuclei were classified according to probability On each drawing, nuclei were counted and the glomerular and the mesangial area de termined planimetrically using a Haff plan meter type no 315 The accuracy of the planimeter itself was frequently checked on a 100 cm² area by means of a control ruler and found to be about 0 1 per cent All areas were measured at least twice If the difference between two measurements of total glomerular area exeeded 1 per cent addi tional measurements were undertaken As regards the individual mesangial areas, more than two measurements were carried out if the first two measurements differed

Using the method comprising *differential counts of nuclei by direct microscopy and determination of the mesangial area by point counting* it was not possible to use oil immersion as the total glomeru lus often failed to be comprised in the field of vision This problem was of no importance in the camera lucida drawing method because both paper and glomerulus could be shifted in parallel Dif ferential counts by direct microscopy were done at a total magnification of × 500 An ocular with a grid was used in order to keep track of the nuclei which were counted on a laboratory counter Mesangial area determination was done by project ing the glomeruli onto a piece of cardboard with points having a space corresponding to 17 μm of the glomerulus The total linear magnification was × 1035 Central sections of the glomeruli were placed in ten positions and the peripheral sections in twenty A central section was defined as a sec tion from the central 50 per cent of the glomerulus All points coinciding with the mesangium were counted and the mesangial areas were calculated on the basis of the mean value of the ten or twenty examinations Points lying on the boundary of the mesangium were counted as half

During preparation of the point count method the principles for conducting a systematic point count were followed (*Hilliard* 1968, *Underwood* 1970) The number of mesangial areas (αphase areas) was on the average, known from the camera lucida drawings As the points should be

spaced in such a way that about one point on the average falls on any α phase area (*Hilliard & Cahn* 1961), a point spacing corresponding to about 17 µm of the specimen was found to be adequate The decision to count the mesangium in ten fields (i e positions), using central sections was based on a test of ten glomeruli Each glomerulus was placed in ten fixed positions and the standard error of the mean (SEM) of the ten Pα of the individual glomeruli was on an average, found to be <10 per cent of x̄ a result that was found acceptable with regard to statistical precision and economy of time (Pα = total number of points coinciding with the phase α) Using peripheral sections the mesangium should be examined in about twenty positions in order to achieve a precision similar to that obtained in the central sections

Point counting was performed on projected glomeruli as this was found to cause less eye strain than direct counting in the microscope

Measurement of *the total glomerular area* was done in four ways 1) planimetrically 2) by point counting 3) determining the area as an ellipse and 4) determining the area as a circle Planimetric measurement was done as described under the camera lucida drawing method Using the point count method the point spacing was the same as that used for mesangial area determination but points were counted only in three positions This was done because a test of ten glomeruli revealed that the average SEM of the individual glomeruli was <2 per cent of x̄ if counted in three positions Determinations of the glomerular area, applying the formula of an ellipse or a circle, was done by measurement of the two greatest right angled diameters or average radius, respectively

In the auto blind studies the results of the first examination were filed before the second examination was performed The time interval between two examinations was 1 month when the camera lucida drawing method was used and 1 week when direct microscopy and point count were used The difference in time interval between the two examinations was determined by the fact that details of nuclear and mesangial area identification could be remembered for a longer period of time if the former method was used

All examinations in this study were made by one of us (FHS) with the exception of the planimetric measurements which were carried out by a technical assistent

RESULTS

Total- and differential counts of nuclei The reproducibility of the total and differential counts of nuclei using the camera lucida drawing method and the direct microscopy, was evaluated by auto blind studies Ten

TABLE 1 *Total and Differential Counts of Nuclei Camera Lucida Drawing An Auto Blind Study of Ten Glomeruli*

Glomerulus no	Total nuclei			Mesangial nuclei			Endothelial nuclei			Epithelial nuclei		
	Number		Difference	Number		Difference	Number		Difference	Number		Difference
	A	B	A – B	A	B	A – B	A	B	A – B	A	B	A – B
1	111	114	–3	26	27	–1	43	45	–2	42	42	0
2	124	128	–4	33	33	0	55	54	+1	36	41	–5
3	121	118	+3	40	39	+1	53	52	+1	28	27	+1
4	136	134	+2	51	51	0	49	45	+4	36	38	–2
5	139	138	+1	38	36	+2	57	58	–1	44	44	0
6	136	138	–2	45	49	–4	62	63	–1	29	26	+3
7	106	108	–2	32	34	–2	38	38	0	36	36	0
8	102	99	+3	26	24	+2	43	44	–1	33	31	+2
9	158	158	0	52	49	+3	72	74	–2	34	35	–1
10	156	154	+2	47	46	+1	64	63	+1	45	45	0
Total	1289	1289	0	390	388	+2	536	536	0	363	365	–2
IEM %			1 3			3 6			3 3			4 1

A = first examination B = second examination IEM % = index of error of measurements per cent = $\frac{SD_{difference}}{\bar{x}_{numbers}} \times 100$

$SD_{difference}$ = standard deviation of differences (A — B) x̄ = mean

TABLE 2 *Total and Differentiol Counts of Nuclei Direct Microscopy An Auto Blind Study of Ten Glomeruli*

ne-s	Total nuclei			Mesangial nuclei			Endothelial nuclei			Epithelial nuclei		
	Number		Difference	Number		Difference	Number		Difference	Number		Difference
	A	B	A–B	A	B	A–B	A	B	A–B	A	B	A–B
	122	120	+2	35	35	0	47	47	0	40	38	+2
	94	90	+4	24	24	0	45	43	+2	25	23	+2
	54	54	0	10	12	—2	22	22	0	22	20	+2
	90	93	—3	23	22	+1	40	44	—4	27	27	0
	104	106	—2	20	23	—3	57	56	+1	27	27	0
	105	111	—6	27	30	—3	52	53	—1	26	28	—2
	85	86	—1	23	22	+1	41	43	—2	21	21	0
	107	111	—4	26	31	—5	50	47	+3	31	33	—2
	76	82	—6	16	17	—1	37	42	—5	23	23	0
	88	93	—5	22	23	—1	33	36	—3	33	34	—1
al	925	946	—21	226	239	—13	424	433	—9	275	274	+1
I %			2,8			6 8			4,4			3 7

A = first examination B – second examination Definition of IEM % see Table 1

glomeruli used for camera lucida drawing and another ten glomeruli used for direct microscopy were examined twice The results are seen in Table 1 and 2 Both methods show good reproducibility, but if the two methods were compared, the indices of erorr of measurements (IEM) involved in the camera lucida drawing method were, on an average, found to be lower

Measurement of mesangial area The glomeruli examined were the same as those used for total differential counts of nuclei

The reproducibility of the camera lucida drawing method depends on the precision of the planimetric procedure and on the precision of determination and drawing of the boundaries of the areas The precision of the planimetric procedure was tested by measuring the *same drawing twice* (Table 3), and the reproducibility of the drawing plus the planimetric procedure was tested by measuring *two drawings of the same glomeruli* (Table 4) Comparison of the precision of the planimetric procedure and the precision of the camera lucida drawing method shows that the most important cause of experimental error is the difficulty involved in determination and drawing of the boundaries of the mesangial areas

Using systematic point count for measurement of mesangial area the reproducibility of the method was found to be good (Table 5), and by comparison of systematic point count and camera lucida drawing the former method was found to be more precise than the latter for the determination of mesangial area (Table 4 and 5)

Measurement of total glomerular area Ten glomeruli chosen without regard to size and shape were used for a comparison of the above described four methods for determination of total glomerular area Using all four methods an auto blind study of the ten glomeruli was undertaken and good reproducibility, which applies to all methods, was revealed The IEM per cent of the four methods calculated on the basis of the differences of 2×10 determinations, was as follows 1) 0 5 (camera lucida drawing method), 2) 1 1 (point count method), 3) 1 8 (diameter method applying the formula of an ellipse) and 4) 1 4 (diameter method applying the formula of a circle) The results obtained by the four methods used for deter-

TABLE 3 *Precision of the Planimetric Procedure in Determination of Mesangial Area*

Glome rulus no	Mesangial area μm^2 AI	AII	Diff I–II
1	1132	1192	— 60
2	1856	1928	— 72
3	1756	1808	— 52
4	2560	2472	+ 88
5	1968	2004	— 36
6	2708	2748	— 40
7	1380	1396	— 16
8	1420	1420	0
9	2344	2244	+100
10	2216	2256	— 40
Total	19340	19468	—128
IEM %			2 1

Ten drawings measured twice AI = first examination AII = second examination IEM % = index of error of measurements per cent = $\frac{SD_{difference}}{\bar{x}\ mes\ areas} \times 100$

$SD_{difference}$ = standard deviation of differences (I—II)

$\bar{x}$ = mean

TABLE 4 *Determination of Mesangial Area Camera Lucida Drawing (Drawing + Planimetric Procedure) An Auto Blind Study of Ten Glomeruli*

Glome rulus no	Mesangial area μm^2 A	B	Diff A B
1	1132	1204	— 72
2	1856	1752	+104
3	1756	1496	+260
4	2560	2188	+372
5	1968	1788	+180
6	2708	2756	— 48
7	1380	1432	— 52
8	1420	1252	+168
9	2344	2232	+112
10	2216	2332	—116
Total	19340	18432	+908
IEM %			6 6

A = first examination B = second examination Defin tion of IEM % see Table 3

TABLE 5 *Determination of Mesangial Area Point Count Method An Auto Blind Study of Ten Glomeruli*

Glome-rulus no	Mesangial area μm^2 A	B	Diff A B
1	1467	1527	— 60
2	1301	1180	+121
3	771	726	+ 45
4	1346	1270	+ 76
5	1422	1497	— 75
6	1180	1089	+ 91
7	1255	1285	— 30
8	1180	1194	— 14
9	1013	1013	0
10	1180	1301	—121
Total	12115	12082	— 33
IEM %			4 4

A = first examination B = second examination Definition of IEM % see Table 3

mination of total area were compared by means of a t test for compared comparison (Table 6) Comparison of the camera lucida drawing method and the point count method revealed no significant difference, but comparison of the camera lucida drawing method and the diameter method applying both the formula of an ellipse and the formula of a circle showed that the results of the camera lucida drawing method were significantly smaller (in both cases $p < 0\,01$)

The relationship between method and time The above mentioned results concerning the precision of the methods employed do not clarify whether the camera lucida drawing method or direct microscopy and systematic point count should be used for quantitative studies of glomeruli, as the reproducibility of both methods is good With regard to differential counts of nuclei, the camera lucida drawing method is the more precise whereas the point count method is the more precise as regards determination of the mesangial area The method of choice should therefore be the more rapid one

Two glomeruli measuring approximately

TABLE 6 *A Comparison of Four Methods for Determination of Total Glomerular Area*

	Total area μm²			
	I	II	III	IV
No	CLDM	PCM	$\frac{a \times b}{4}\pi$	πr^2
1	8694	8576	9811	10562
2	13062	13158	14289	14575
3	13804	14253	14493	14575
4	12698	13158	12979	13358
5	9464	9482	10212	10903
6	17766	18317	18228	20722
7	9443	9609	10079	10718
8	14665	14640	15212	15620
9	13727	13738	14159	14234
10	9716	9675	10219	11693
	12304	12461	12968	13696

CLDM = Camera lucida drawing method
PCM = Point count method
$\frac{a \times b}{4}\pi$ = Area of an ellipse
πr^2 = Area of a circle
Comparison of I and II $p > 0.05$
Comparison of I and III $p < 0.01$
Comparison of I and IV $p < 0.01$

19000 and 24000 μm, respectively, were examined by both methods with a view to estimating the factor of time It took about 80 minutes to make one drawing followed by planimetric area measurements whereas the duration of one examination comprising direct microscopy and point counting was about 20 minutes The difference in time was not equally excessive when smaller glomeruli were examined but the camera lucida drawing method was on the average, found to be at least three times more time consuming than direct microscopy and point counting

None of the methods used for determination of the total glomerular area are time consuming, but measurement of the diameters is the quickest and the camera lucida drawing method the slowest, taking about 1.5 and 3 minutes, respectively, for determination of one central glomerular section The method of choice should, however, be the camera lucida drawing method, or the point count method because of the above described statistical findings, and because the diameter methods can be only approximative owing to the variations in glomerular shape

Examination of 10 normal glomeruli Central and random sections of 10 glomeruli from the same tissue specimen were examined quantitatively The selected glomeruli were consecutive glomeruli from one end of the tissue specimen As seen in Table 7, the standard diviation (SD) of all parameters was greater when random sections were examined than when central sections were examined Using central sections, the SEM of all parameters was $\gtreqless 5$ per cent of $\bar{x}$ a result that we have found acceptable with regard to statistical precision From the formula $SEM = \frac{SD}{\sqrt{n}}$ it was calculated, by comparison of the results of central and random sections that about 30 random sections should be examined if a statistical precision more or less equal to that obtained by examination of 10 central sections were to be obtained

DISCUSSION

General use of quantitative methods for an investigation of glomeruli depends upon the availibility of a satisfactory technique and on

TABLE 7 *A Comparison of ten Central*

	Total area μm	Mesangial area % of total area	*Total nuclei*	Mesangial nuclei % of total nuclei
Central sections	15718	10.6 (1.3)	104	26 (3.3)
Random sections	12325	10.4 (2.1)	76	26.1 (5.0)

Standard deviations are in parentheses

the number of glomeruli needed in a tissue specimen to be examined

Essential for a suitable method is acceptably consistant results of the examinations and, furthermore the method should not be too time-consuming As to differential counts of nuclei and determination of the mesangial area, the reproducibility of both methods used in this study was found to be good A similar good reproducibility of the camera lucida drawing method has previously been reported by *Kawano et al* (1969) Direct microscopy and point counting is, however, superior as to speed and, for this reason it should be used instead of the camera lucida drawing method

In this study, determination of the total glomerular areas planimetrically and by point counting revealed no statistically significant differences in the results, whereas the results of the camera lucida drawing method differed significantly from those obtained by measurement of the diameters applying the formula of an ellipse or a circle *Conte & Mignon Conte* (1968) have previously compared total glomerular areas determined planimetrically and as an ellipse, and found no statistically significant differences in the results obtained by the two methods The divergence in the results of the two studies can possibly be explained by differences in the shape of the glomeruli examined Recently *Wehner et al* (1971) determined the total glomerular area as a circle, but no comparison with other methods for total area measurement was made Because of variance in glomerular shape, determination of the total glomerular area, using the formula of an ellipse or a circle, will be only approximate For this reason and because of the statistically significant differences found in this study, we would prefer in the future to use point counting or the planimeter for total area determination

Using only central sections, a number of about ten glomeruli per tissue specimen seems to be a reasonable number for quantitative examination About three times as many glomeruli should be examined, however, if random sections are used, in order that the same statistical precision may be obtained This is in agreement with the previous finding, namely that the values of the relevant parameters are relatively constant only in the central portion of a glomerulus (*Hanberg Sorensen* 1972) Because many renal biopsies do not contain thirty intact glomeruli, we find it advisable to use only central sections of glomeruli We are, however, aware of the fact that the periphery of the tufts is comparatively less represented in central sections than in random sections

Wehner used a microscope with a binocular tube for differential counts and a Visopan Reichardt for point counting, whereas we used only one ' apparatus , namely a microscope with a monocular tube and a mirror for projection Each of the techniques has practical advantages and disadvantages, and one of them is apparently no more recommendable than the other The point counting was, however in both techniques applied to the projected glomeruli and not directly in the microscope, as the examination was found to be less staining in the first mentioned way (*Wehner* 1971) As regards the point

and ten Random Glomerular Sections

Endothelial nuclei % of total nuclei	Epithelial nuclei % of total nuclei	Total nuclei per 1000 μm² of total area	Mesangial nuclei per 1000 μm² of mesangial area
43 9 (3 0)	30 1 (4 8)	6 6 (0 7)	16 2 (2 1)
39 8 (5 4)	34 0 (6 4)	7 0 (0 9)	17 7 (3 6)

spacing it was about the same in the studies by Wehner and in this study, namely 15 and 17 μm

On the basis of the results of this study we find it advisable to use the following method for quantitative studies of glomeruli which include total and differential counts of nuclei and determination of total glomerular and mesangial areas Direct microscopy for total and differential counts of nuclei, systematic point count for mesangial area determination and planimetric measurement or systematic point count for determination of the total glomerular area The method should be applied to central glomerular sections

ADDENDUM

After the completion of this paper we have received the results of the last quantitative investigation carried out by *Kimmelstiel* and his group (*Kawano et al* 1971) It is a study of methodology, comparing camera lucida drawing and point counting in determinations of mesangial and total glomerular area Camera lucida drawing was found to be the preferable method for area determination as well as for differential counts of nuclei An attempt to do differential counts of nuclei by direct microscopy was abandoned because of too much variation in the results obtained by different observers

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This study was supported by a grant from the *Danish Medical Research Council*

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Acta path microbiol scand Section A 80, 729–735, 1972

CARDIAL COLLOID

Frequency, Histochemical Nature and Relation to Amyloid

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An unselected autopsy series of 71 patients was investigated for the occurrence of cardial colloid The change was found in 51 per cent of the cases (36 patients) The different types of the morphology and histochemistry of cardial colloid are described A similarity between the chemical composition of cardial colloid and amyloid was found, and possibly the pathogenesis of both substances has much in common In one fourth of cases of cardial colloid small amyloid precipitates were found in the alveolar septa and arteries of the lungs As cardial colloid more often is found in old age the change possibly bears relationship to senile amyloidosis

Cardial colloid (C C) (10) is a mucinous change in the myocardium consisting of homogeneous, granular or vacuolated focal deposition of basophilic material in the muscle fibres often close to the sarcolemma

Therefore, this pathological alteration has also been termed basophilic degeneration (4, 9 and 18) As apparent from the belowmentioned results, however, this terminology is less significant than terms like mucoid degeneration (4, 21 and 22) and mucinous degeneration (18) In the present paper the term cardial colloid is used as a neutral term covering the whole spectrum of this alteration in cardiac muscle

C C was described by *Geipel* 1905 (7) who found blue areas in the myocardial cells in haematoxylin eosin stained preparations from a 16 years old girl who died from rheumatic myocarditis C C was later investigated by others (2, 4, 9, 10, 14, 15, 18, 21 22) Nevertheless, although a common finding, C C is often ignored or overlooked in autopsy series, as stated by *Rosai* & *Lascano* (17)

Some investigators have found a connection between the occurrence of C C and malignant neoplasms, cardiovascular, cerebrovascular and renal diseases (9, 18) Others have observed a relationship between C C and thyroid disease, especially myxoedema (2, 4, 10 14, 22), idiopathic hypertrophic cardiomyopathy (6) and progressive familiar myoclonic epilepsy (8) *Ebbesen et al* 1970 (5) found a relatively high frequency of myocardial lesion much resembling human C C in mice bearing transplanted plasma cell leukaemia with concomitant kidney and spleen amyloidosis

Another important relationship between C C and amyloid substance was found in electronmicroscopic investigations by *Kosek* & *Angeli* (13) and *Rosai* & *Lascano* (17) These investigators found C C characterized by a framework of fibrils 70–100 Å in diameter and, in addition, granules 150 to 3–600 Å in diameter The amyloid fibrils are 75–80 Å in diameter (19) and a component

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of granular material is also found (3) Whereas the C C was found constantly in intracellular location (13 17), amyloid intracellularly has only been reported in a few papers (11, 12, 16, 20)

The aim of the present work has then been to further elucidate the possible connection between C C and amyloidosis in man Special attention was paid to the simultaneous occurrence of C C and senile cardiovascular amyloidosis which may be regarded as a special form of primary amyloidosis predominantly localized to the heart in patients more than 70 years old (3) In such a case, *Beneke & Böning* 1908 (1) also found amyloid in the pulmonary veins and in the lungs This comparative study was done by comparison of the results of histochemical reactions and by investigating the coincidence of the two conditions in an unselected autopsy series

MATERIAL AND METHODS

The total series comprised 71 consecutive autopsies The investigations were retrospective using 1 to 6 tissue specimens from different parts of the heart, mostly from the left ventricle Fixation was done with buffered formalin at pH 7,2, concentration 10 per cent for 24 to 72 hours Paraffine embedding was used and routine sections cut 6–8 μ thick and stained with haematoxylin eosin Additional staining techniques were periodic acid Schiff reaction (PAS) for vicinal hydroxyl groups especially neutral mucopolysaccharides or glycoproteins for demonstration of acid mucopolysaccharides was used toluidine blue 0 1 per cent at pH 3 0 Mowry s alcian blue technique at pH 2,5, and the colloidal iron staining a m Hale in Mowry s modification For fibrin or fibrinoid substance was used the Frazer Lendrum staining technique with picro Mallory and Mallory s phosphotungstic acid haematoxylin (PTAH) matured for months The last mentioned method was also helpful for the demonstration of striation in the muscle tissue The alkaline Congo red method of Puchtler, Sweat & Levine was used for identification of amyloid substance followed by examination in polarized light to show the green dichroism (or birefringence) specific for this alteration Some myocardial sections were stained with long Ziehl Neelsen technique and thionin pH 3 and Sudan black B as it has been claimed, by some authors that this was a means by which to distinguish between lipofuscin (positive) and C C (negative) For the same reason autoflourescence was investigated lipofuscin being characterized by this property The method included determination of the localization of C C by Haem Eos staining and after decolorization investigation of the positive areas by fluorescence microscopy, and determination of the localization of C C of unstained sections and finally control staining of these with PAS Besides sections from the heart, one or more sections were taken from the lungs liver, spleen and kidneys and from pathological processes observed by gross examination or found to be appropriate to the clinical findings They were all investigated for the presence of amyloid change

RESULTS

Among the 71 autopsies, C C was found in 36 patients This finding of C C in the present series could be divided into two distinct and separate groups

Group I represented 21 patients In these C C was found in Haem Eos-stained preparations as bluish grey often finely granulated masses in perinuclear position The colloid might also appear as fragmented masses or more seldom as a homogeneous hyalin mass In every case, the sarcolemma was preserved peripherally as a thin rim In all instances the C C reacted PAS positively In 16 of these cases, the C C was also positively stained in the colloidal iron staining and 12 cases showed a positive alcian blue reaction Toluidine blue showed no positive reaction

Group II consisted of 15 patients By careful microscopical examination of the Haem Eos stained preparations a perinuclear clear zone of the appearance of a pale network composed of large meshes was observed These areas were very clearly demonstrated in PAS staining, using this method, the degeneration had just the same morphological picture as in group I In group II, however only 5 patients showed a positive colloidal iron reaction and alcian blue reactivity was demonstrated in a single case As in group I, there were no positive reaction for toluidine blue

Among the patients in the above mentioned groups I and II, 9 cases were found to

present tiny amyloid precipitations in the alveolar septa of the lung and, in addition, amyloid substance in the walls of the branches of the pulmonary arteries In 3 cases, amyloidosis of the heart without any direct relationship to the presence of C C was found morphologically In these cases, the amyloid change was observed in walls of arteries and in 2 cases, also interstitially in the myocardium In one single case, amyloid was also demonstrated in the vessel walls of the perirenal tissue

Group III ('control group) was composed of the remaining 35 autopsies in which C C was not detected Amyloid was demonstrated in one case, namely in the pancreas

C C was not 'acid fast in long Ziehl Neelsen staining and did not stain with thionin and Sudan black B, it did not show the autofluorescence, *i e* it did not show these properties which lipofuscin in general is said to exhibit

Table 1 shows the correlation between the age of the patients and the pathological findings of C C and of amyloidosis

The staining techniques used for fibrin and fibrinoid were negative in all cases (with the exception of the PAS staining)

DISCUSSION

The juxtanuclear position of C C, the positive PAS reaction and the demonstrated increasing frequency with age, are in a way characteristics comparable to those of lipofuscin However, between these two conditions there are pronounced morphological differences Brown atrophy' (lipofuscin) has another appearance than that described here for C C by being yellowish brown of finely granulated character, and it is 'acid fast', stainable with thionin and Sudan black B and it is autofluorescent The histochemical investigations of C C in the past have given different opinions about the chemical nature of this pathological substance within myocardial fibres *Geipel* (7) thought it might be derived from the cell nucleus but several investigators (15 17 and 18) have argued against this assumption Others have proposed C C to be a mucinous substance presumably linked to glycogen (9), mucoprotein or acid mucopolysaccharides (15 and 18)

Our present work points to a content predominantly of neutral mucopolysaccharides or glycoprotein by the positive PAS-reaction In a considerable number of cases, however, there is often an admixture of acid mucopolysaccharides, the exact nature of which has not yet been thoroughly investigated, as demonstrated by the positive reactions with the colloidal iron Hale method and the alcian blue positivity Recent histochemical, physico-chemical (17) and electron microscopic investigations (13 and 17), however, seemed to indicate that C C was glucose polymers rather than the above-mentioned neutral and acid mucopolysaccharide complexes with protein

Indeed, there is a considerable similarity between the staining results obtained for C C and amyloid substance (compare *Christensen* (3)) In amyloid substance, a central core of PAS positive substance is often found indicating the presence of glycoprotein but peripherally in the precipitates, positive colloidal iron-, alcian blue

Fig 1 Bl ish—grav or basophilic staining of C C in hear muscle s a ned with Haem Eos

Fig 2 Clear pe nuclear zone showing vacuolated appea ance of C C in Haem Eos stained section

Fig 3 Heavily PAS stained large C C formation occupying the whole muscle fibre

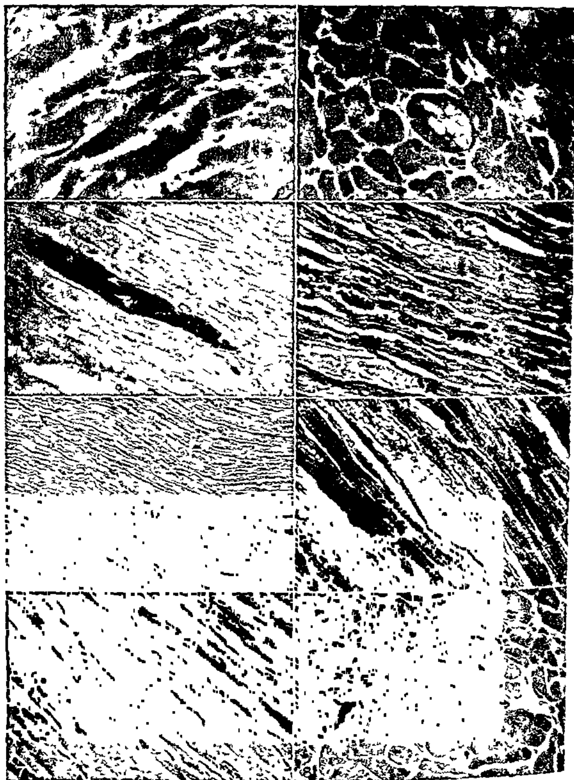
Fig 4 C C showing positive alcain blue reaction

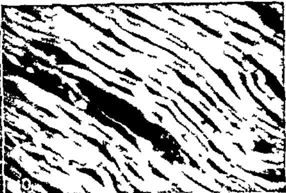
Fig 5 C C in centre unstained in Lendrum staining for fibrin

Fig 6 C C in centre unstained in PTAH staining

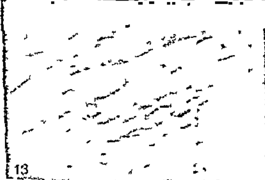
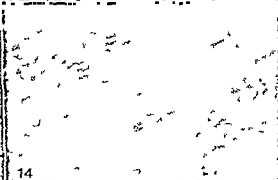
Fig 7 Long Ziehl Neelsen stained heart muscle showing red granules of lipofuscin and unstained C C in centre

Fig 8 Heart muscle stained with Sudan black B showing black stained lipofuscin granules and unstained C C of vacuolated type in centre




9


11


13

14

15

16

TABLE 1 *Schematic Tabulation of the Patients in Different Age Groups Concerning Negative and Positive Findings of Cardial Colloid (C C) Pulmonary (P A) and Cardial Amyloid (C A)*

Age in years	—C C —P A —C A	+C C —P A —C A	+C C +P A —C A	+C C +P A +C A	+C C —P A +C A	Total
0–19	10	0	0	0	0	0
20–39	3	0	0	0	0	0
40–59	5	6	0	0	0	6
60–79	11	16	2	2	0	20
≧ 80	6	5	4	1	0	10
Total	35	27	6	3	0	36

and metachromasia techniques point to the presence of acid mucopolysaccharides These latter substances might be due to a secretory reactivity of surrounding reticular tissue cells upon an initial genuine deposition of pre-amyloid substance (glycoprotein) or a definitive unsoluble precipitation of such

The similarity between the fibrillary composition of C C and amyloid was mentioned in the introduction This together with the histochemical findings point to a pathological relationship which perhaps also is reflected in the pathogenic processes responsible for these changes This assumption seems to be further established by the simultaneous occurrence of both C C and small amyloid precipitates in lung alveolar septa and walls of small lung arteries in 1/4 of the patients, whereas the control group was negative Perhaps the cardial colloid of old age and senile cardiovascular amyloidosis are related conditions

Fig 9 C C demonstrated in H E stained section (compare Fig 10)

Fig 10 Autofluorescence of the same preparation as Fig 9 after decoloration The heart muscle fibres show light green fluorescence whereas lipofuscin appears yellow—and C C is nonautofluorescent

Fig 11 Unstained preparation showing the same results as Fig 10

Fig 12 Same section as Fig 11 after staining with PAS Now C C is clearly demonstrated

Fig 13 Amyloid demonstrated in heart with alkaline Congo red

Fig 14 Same as Fig 13 in polarized light showing green di-croisme of amyloid

Fig 15 Amyloid demonstrated in alveolar septa of lungs with alkaline Congo red

Fig 16 Same as Fig 15 in polarized light Magnification for all illustrations × 250

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Acta path microbiol scand Section A 80, 736–742, 1972

THE DIURNAL VARIATION OF THE FIRST MITOTIC WAVE RELEASED AMONG RAT KIDNEY TUBULE CELLS BY PARTIAL NEPHRECTOMY

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Mitotic waves were released in kidney tubules by resecting one kidney and a half in a group of 4 rats at intervals of a few hours during the 24 hr cycle The course of the mitotic wave appearing on the second day after the operation was followed in each half kidney by determining the incidence of mitosis at intervals of 3 hours The waves showed a distinct diurnal pattern of variation those initiated by resection between noon and midnight had single peaks while those initiated by resection between 3 a m and 9 a m gave waves with 2 peaks It is assumed that the single as well as the paired waves are based mainly on mitoses in the proximal tubules The alternation between sequences of single and double peaked waves through the 24 hours is interpreted as the result of an interference of an endogenous mitotic rhythm the mitotic wave starting slightly less than 30 hr after the resection

The mitotic activity of many mammalian tissues shows a distinct diurnal variation The timing and the relative amplitude of the maximal mitotic activity depend on the kind of tissue considered In certain tissues a single, large maximum is produced during the 24 hr *e g* in the liver (1), the inter-alveolar septa of the lung (14) and the abdominal epidermis of the young rat (4) In the adrenal cortex it has been shown that the diurnal mitotic rhythm of the zona glomerulosa and the zona fasciculata has an almost opposite pattern (17) A more irregular variation with several mitotic maxima during the 24 hr cycle has been observed in other tissues, *e g* in the crypts of Lieberkuehn (11) and in the ear epidermis of the male mouse (6) With respect to tissues which maintain the cell population of the blood the observations are more contradictory some workers conclude that the mitotic activity of the bone marrow (10) and the lymph nodes (12) show a diurnal variation while the results obtained by others indicate that the mitotic index remains unchanged through the 24 hr period (8 5)

The present study deals with the diurnal variation of the large mitotic wave that is initiated in the kidney tubules on the second day after the resection of one kidney and a half Four rats were resected every 3 hours through the 24 hr cycle, and the course of the mitotic wave was determined in each animal As a distinct diurnal rhythm of the normal

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mitotic activity in the convoluted tubules has been demonstrated (3, 15, 18), some variation in the course of that wave depending on the hour of operation may be anticipated

METHODS

The rats belonged to an outbred stock of albino rats that had been maintained for several decades at the Institute They were raised on commercially available food pellets intended for chicken In addition, the rats had access to whole grain bread, fresh, whole milk Swedish turnip and water *ad libitum* After weaning, the animals were kept in a large cage the top of which was covered by a coarse wire netting In the cage was a dark, wooden enclosure to which the rats had free access through a small opening in the side Five weeks before the vernal equinox the cage was placed in a quiet room beneath a large window in the ceiling During the night, no reflection from electric light could reach the animals through this window and no electric lighting was ever used in the room Due to the relatively high latitude of Oslo the transition between day and night is very gradual The temperature of the room varied between 19 and 22° C Food was replenished and the cage cleaned between 5 and 6 p m

Partial nephrectomies were performed, beginning at the vernal equinox and continuing through the following 3 weeks At this time the rats were slightly more than 12 months old and their ages varied by less than 2 weeks The entire right and the caudal half of the left kidney were removed simultaneously according to a schedule which during the course of the 3 weeks gave 4 resected rats every 3 hr through the 24 hr cycle Details of the operation procedure and the method of sample taking as well as the preparation of the tissue specimen for the determination of the incidence of mitosis are described elsewhere (16) The incidence of mitosis is defined as the sum total of all the stages of dividing tubule cells contained in one milligram of cortical tissue The samples were excised from the sides of the kidney and included cell material to a depth of 0 8 mm in the cortex Four samples were taken from each side of the half kidney providing 8 consecutive readings of the incidence of mitosis for the plotting of the mitotic wave The peak of the wave gives the maximal incidence of mitosis It was known from earlier work (16) that the first mitoses appeared slightly less than 30 hr after the diminution of the kidney mass Therefore 36 hr after the operation was considered a suitable time at which to excise the first tissue specimen in each of the series of sample-takings The first 5 samples were taken at intervals of exactly 3 hr while up to 10 hr were allowed to pass between the excision of the 3 last samples These latter tissue specimens were intended for the detection of a possible second mitotic wave during the third postoperative day

RESULTS

Fig 1 shows the mitotic waves which appeared on the second day after the resection of a kidney and a half every third hour during the 24-hr cycle Sometimes a smaller wave also appeared on the third day after the operation Each wave represents the average of the 4 waves obtained in the half-kidneys from 4 animals operated at the hour indicated The only exception is the 3 p m -wave, *i e* the wave initiated by operation at 3 p m In one of the animals resected at this hour, the wave was disproportionately small, much smaller than the other 3, and was therefore omitted

The 12 noon-wave Partial nephrectomy performed at 12 noon consistently released two consecutive waves a large wave followed by a very small one The maximal incidence of mitosis appeared 43 hr and 65 hr after the resection The minimum occurred close to 52 hr after the resection The course of the ascending slope of the first wave indicated a preceding non mitotic period of slightly less than 30 hr

The 3 p m and 6 p m -waves These waves were very similar to the 12 noon-wave with respect to their over-all shape, their maximal height, and the point of time after the operation at which the maxima were produced

The 9 p m -wave This wave differed markedly from the preceding waves it appeared as a single, evenly descending slope starting with its highest incidence of mitosis 36 hr after the operation No small wave appeared on the third postoperative day

The 12 midnight wave The resections performed at midnight produced 4 waves with the same general course as the 12 noon-, 3 p m - and 6 p m waves a high, but very sharp peak with a maximum about 38 hr after the resection, was followed by a very low peak on the third postoperative day

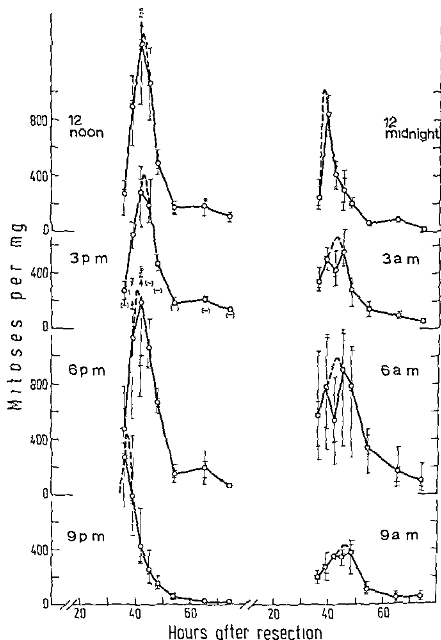


Fig 1 The variation of the mitotic waves through the 24 hr-cycle Each wave represents the average of the 4 experimental waves initiated by resecting a kidney and a half at the hour shown The incidence of mitosis of the experimental waves are indicated

The 3 a m -, 6 a m - and 9 a m -waves The top of these waves differed markedly from that of the preceding waves by being split into 2 peaks The minimum incidence of mitosis between the peaks of the 3 a m - and 6 a m -waves appeared about 42 hr after the operation while the minimum of the 9 a m - wave occurred about 3 hr later No low peak appeared on the third day after the operation The double-peak appeared in each of the 4 waves which were averaged to produce the 3 a m - and the 6 a m -waves The period of time between the 2 maxima covered a little more than 6 hr Two of the experimen-

tal 9 a m waves were double peaked, while the other 2 had a single but flattened top Except for the splitting of the peak the general course of the 3 a m , 6 a m and 9 a.m waves was similar to that of the single peaked waves produced by operation between noon and midnight

In addition to the shift between single and double peaked waves during the 24 hr cycle the period of time between the operation and the attainment of the maximal incidence of mitosis also changed with the hour of operation To disclose the pattern of this variation the waves were arranged according to the points of time at which their maxima were produced In Fig 2 the vertical lines indicate 12 midnight and the dashed lines 12 noon

The single peaked waves appearing on the second postoperative day which were released by resections between noon and midnight attained their maxima approximately at 7 a m 10 a m 11 a m 9 30 a m and at 2 p m respectively This means that the top of these 5 waves was produced within the total span of time of only 7 hr with a tendency to cluster around noon The 3 double peaked waves released by the operations performed at 3 a m. 6 a m and 9 a m produced on the other hand their over all maxima at about 10 p m 1 a m and at 7 a m *i e* they were scattered over a period of 9 hr

DISCUSSION

It has been shown that the mitotic waves concerned are submitted to a distinct diurnal variation with respect to size as well as to shape. Even if the size of the 4 waves initiated by resection at any definite hour may deviate comparatively much from that of the average wave (see Fig 1) the 4 experimental waves maintain an almost parallel course their peaks being produced at nearly the same hour after the resection

The 32 experimental waves have two features in common firstly the waves start after a preceding non mitotic period covering between 24 and 30 hr only the 9 p m wave seems to represent an exception (to be discussed below) Secondly, the maximal incidence of mitosis is attained on the second day after the resection The non mitotic period has been discussed elsewhere (16), one point only will be related here In this earlier study it was found that with increasing size of the waves the period of time between the resection and the attainment of the maximal incidence of mitosis becomes ever shorter This means that the ascending slope rises disproportionately more steeply when the height of the wave increases There is every probability that this phenomenon has a bearing on the exceptional 9 p m wave which according to the incidence of mitosis observed is presented by a descending slope only It may be assumed that the course of this wave fits into the pattern of gradual changes of the single peaked waves appearing on the second postoperative day when the operations are performed at successively later hours If so, a sharply pointed peak between 36 and 39 hr after the resection would be highly probable Accordingly the incidence of mitosis obtained at 36 hr would mark a point on the *ascending* slope of the wave

In the absence of any diurnal variation the mitotic waves should be of approximately the same size and shape, and also the interval between the hour of resection and the maximal incidence of mitosis should be of nearly the same duration Fig 2 shows that all 3 parameters vary Studies of the diurnal variation of the mitotic activity in the normal kidney indicate that the endogenous conditions favour an increased mitotic activity at about noon (15) or in the afternoon (3 18) and correspondingly fewer mitoses before midnight Consequently the way the mitotic waves vary during the 24 hr cycle may be related to an endogenous or diurnal mitotic rhythm Before discussing this problem it is necessary to consider the cellular basis of the conspicuous double peaked waves that appear after operations at 3 a m 6 a m and 9 a m In all three waves the double peak is produced by a single low incidence of mitosis at an hour when the maximum of the total wave otherwise should be expected As men

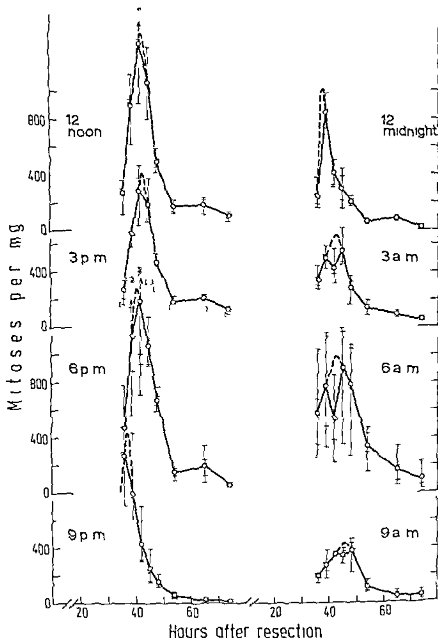


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the proximal tubules, the interval of time between the resection and the attainment of the over all top of the paired waves may be compared This period lasted for 43 hr for the 3 a m waves, the 6 a m waves, and for the single peaked 12 midnight wave Only the over all maximum of the lower double peaked 9 a m wave appeared as late as 47 hr after the resection This produces a wide scattering through the night of the maximal incidences of mitoses for these waves as compared with the clustering of the single peaked waves around noon

The formation of the paired waves may be explained as follows The mitoses initiated by resection during the early and late morning can take place only after the cessation of the non mitotic period This means that the mitotic wave starts late in the evening when the endogenous conditions are less favourable for the onset of mitoses For the 3 a m - and 6 a m wave this internal retardation of mitotic activity increases during the late evening As a result, these mitotic waves pass through a comparatively early maximum close to 39 hr after the resection Meanwhile the impetus to the release of mitoses affected by the resection proceeds so as to reach its maximum when the endogenous inhibition of mitosis is lessened before midnight, immediately following the first peak the mitotic wave rises to a second maximum The less pronounced splitting of the 9 a m wave would mark the decreasing effect of this mechanism after midnight even though the smaller size of this two peaked wave remains unexplained

So far, the discussion is valid only if the duration of the mitosis remains constant through the 24 hr cycle In this case only will the incidence of mitosis be directly related to the fraction of the cells which is entering the mitosis per unit of time *i e* to the mitotic rate It has been demonstrated that after poisoning of the rats kidney by bichromate the mitotic duration of the epithelial cells may change during the regeneration of the proximal tubules (2) Any information concerning a possible diurnal variation of the mitotic duration of these cells is apparently not yet available A variation of this kind has been revealed, however, in the epidermis of mice (7) Because of the very high temperature coefficient of the mitosis (9), the mitotic duration in this surface tissue may be more directly related to the physical activity of the animal than that in an internal organ like the kidney The alternation between sequences of single and double peaked waves given above, could partly be explained by a *corresponding change in the* duration of the mitosis a slowing down of the mitotic time around noon will contribute to the height of the single peaked waves found at this time, while a speeding up of the mitotic process in the middle of the wave during the night could produce the depression that splits the wave into 2 peaks The problem concerning the extent to which the diurnal pattern of variation of the mitotic waves may be due to a different length of the mitotic time will have to await measurements of the latter through the 24 hr cycle

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The Norwegian Research Council for Science and the Humanities is acknowledged for a fellowship given to the author during the work

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EVIDENCE FOR LOCAL RENIN FORMATION IN THE RABBIT OVIDUCT

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The uterus, oviduct, vagina, mesometrium and ovary of mature rabbits all contain renin, by far the highest concentrations being found in the uterus and in the isthmic segment of the oviduct. In contrast to the uterine renin the concentration of the oviduct does not increase significantly during maturity or terminal pregnancy, while oestradiol administration results in a marked increase in both uterus and oviduct. Surgical separation of the oviduct from the uterus results in some decrease, but not disappearance of the renin in the oviduct. Intraocular grafts of oviduct in immature, oestradiol treated and in mature, hysterectomized animals contain renin concentrations equal to or higher than those of the oviduct *in situ*. These findings show that the oviduct contains renin producing cells.

Investigations of the occurrence of renin in the female genital tract of cat (*Stakeman* 1960), rabbit (*Gross et al* 1964, *Bing & Faarup* 1966, *Ferris et al* 1967, *Eskildsen* 1971), dog (*Hodari et al* 1969) and man (*Skinner et al* 1968) have been concerned with the demonstration of renin formation in the uterus and intra-uterine tissue during pregnancy. The finding of renin in not negligible amounts in extra uterine parts of the genital tract, as the vagina and the mesometrium, of pregnant rabbits (*Bing & Faarup* 1966) brings up the question if a production of renin takes place in any other part of the genital tract.

In the present paper a solution of this problem is attempted by comparing the content of renin in the uterus, the oviduct, the vagina, the mesometrium and the ovary of normal, mature rabbits, and by a study of the renin concentration of the oviduct the immature, the mature, and the pregnant state. A closer examination of the possible formation of renin in the oviduct is then performed by studies on the oviduct renin after separation of the oviduct from the uterus, and by intraocular autotransplantation.

MATERIALS AND METHODS

31 female a t io country rabbits, from the State Serum Institute, weighing 1.5–4 kg, were used and divided in 4 main groups. In *Group I*, consisting of 8 normal mature animals, the renin concentration was determined in all the parts of the genital tract including the different sections of the oviduct. In *Group II*, consisting of 6 animals, the renin concentration of the oviduct and the uterus was investigated in 3 immature animals (*identical* with those of Group IV) before and after oestradiol treatment (oestradiol monobenzoate, 10 $\mu g/kg$ daily for 4 days), and in 4 pregnant animals on

Received 5.v.72

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the 28th day of pregnancy The values were further compared with those of 8 mature animals (Group I) In 14 animals from *Group* III the oviduct on the right side was separated from the uterine horn, at the same time cutting the vascular and lymphatic connexion between the mesometrium and the mesosalpinx 7 of these animals were mature and had the two oviducts, the separated and the untouched, removed after 14 days 7 animals were immature at the time of the operation, but mature when the oviducts were removed 70 days later In *Group IV* 6 animals received intraocular autografts of tissue from one oviduct 3 animals were immature, and 14 days after the grafts were implanted on the iris, they received oestradiol monobenzoate (Leo), diluted in sesam oil, injected subcutaneously for 4 days, in doses of 10 μg/kg body weight daily 24 hours following the last injection the grafts, as well as the oviduct and uterus *in situ*, were removed The other 3 animals from this group (IV), which were mature, underwent hysterectomy at the same time as the transplantation was done The ovaries and one oviduct were left intact The grafts and the tissue *in situ* were removed approximately 21 days later

All operations were performed under anaesthesia with pentobarbital sodium (40 mg/kg body weight) Pre and postoperatively the animals were treated prophylactically with penicillin 250 000 I E The final removal of tissues was carried out on animals killed by air intravenously or by a neck stroke

Intra ocular transplantation, placing small tissue flakes of oviduct in the anterior chamber of the eye, was performed as described previously in the case of uterine tissue transplantation (*Eskildsen* 1971) 3 pieces, each measuring 2 × 2 × 3 mm, were placed in one eye of each animal

Biopsies To compare the renin content in the different sorts of tissue of the reproductive organs small biopsies of the vagina and the mesometrium were used, while 2 3 segments of the uterine horn the whole oviduct and the ovary from the same side were taken To compare the renin content in the oviduct on the 2 sides, corresponding pieces with identical distance from the uterus were used For oviduct transplantation the isthmic segment was employed the identical part of the oviduct in situ being used for comparison

Tissue extraction homogenizing and transitory acidification to inhibit angiotensinase activity were performed as mentioned previously (*Eskildsen* 1970)

Renin assay was based on the principle of determination of the decrease in angiotensinogen concentration in the course of time (*Poulsen* 1968) and was combined with a radio immunoassay for angiotensin I (*Eskildsen* 1972) identical in principle with that described for angiotensin II (*Poulsen* 1969) The renin concentration is expressed in Goldblatt Units per gram tissue (GU/g) by reference to a highly purified hog renin standard preparation kindly supplied by Dr Haas and identical with that donated by Dr Haas to the WHO Laboratory for Biological Standards (Nat Inst Med Res, Mill Hill, London)

Histological examination was carried out on tissue fixed in Helly's solution for 24 hours, paraffin embedded, cut in 4 micron thick sections and stained by haematoxylin eosin Van Gieson and PAS, cold procedure (*Petri* 1968)

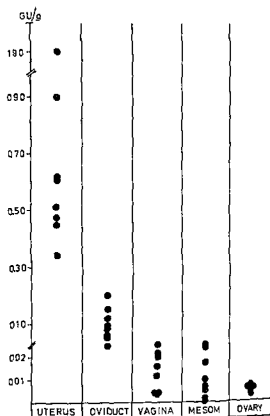


Fig 1 The renin concentration GU/g, of the uterus oviduct, vagina, mesometrium (MESOM) and ovary from 8 mature rabbits The values below 0 025 GU/g are shown in a special scale For comparison the plasma renin concentration *in normal* rabbits is about 1×10^{-4} *GU/ml*

RESULTS

1 *Renin in the Different Parts of the Genital Tract of Female, Mature Rabbits*

In 8 normal, mature animals (*Group I*) the renin concentration of the uterus, the oviduct, the vagina, the mesometrium and the ovary was measured (*Fig 1*) Among

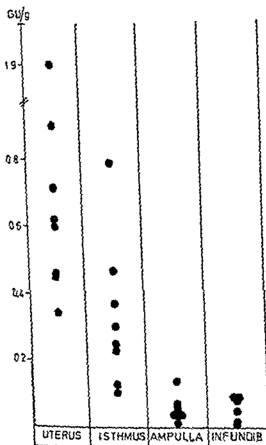


Fig 2 The renin concentration of the three segments of the oviduct isthmus ampulla infundibulum (INFUNDIB) compared with the renin concentration of the uterus from 8 mature animals

these, the uterus has the greatest content of renin and the renin concentration of the oviduct (range 0 026–0 200 GU/g) was markedly elevated above the concentration in the vagina mesometrium and ovary (significance level $p = 0.002$) which had about the same renin content (range 0 003–0 037 GU/g) Expressing, in each animal, the renin concentration of the oviduct vagina mesometrium and ovary in per cent of the renin concentration of the corresponding uterus (Table 1) the renin of the oviduct made up about 15 per cent of the uterine renin while the renin of the vagina, mesometrium and ovary only constituted 1 0–2 5 per cent

After division of the 100–140 mm long oviduct from 8 normal, mature animals (Group I), into the 3 anatomical segments, the 10–20 mm long *isthmus*, connected with the uterus, the 80–100 mm long *ampulla*, and the *infundibulum*, the funnel shaped segment close to the ovary, comparison of the renin content in these fractions were made (Fig 2) The renin concentration of the isthmus was in all cases much higher than that of the remaining parts of the oviduct (significance level $p < 0.001$) The renin concentration of the isthmus was approximately 50 per cent of that of the uterus (Table 1), while those of the ampulla and the infundibulum were only about 10 per cent

TABLE 1 *Renin Concentration in the Female Genital Tract Expressed in Per Cent of the Renin Concentration of the Uterus*

	Tissue	Mean %	S D %	n
Oviduct	Infundibulum	*8 1*	6 8	7
	Ampulla	*7 8*	3 7	8
	Isthmus	*46 4*	17 1	8
	In total	*14 7*	8 6	8
Vagina		*2 5*	2 4	8
Mesometrium		*1 6*	1 0	8
Ovary		*0 9*	0 3	7

In each animal the renin concentration determined in GU g of the different sorts of tissues was expressed in per cent of the renin concentration of the uterus

2 *Renin Concentration in Oviduct During Oestradiol Treatment and Pregnancy*

In 3 immature animals, the renin concentration of the oviduct and the uterus was investigated before and following 4 days treatment with oestradiol (oestradiol monobenzoate, 10 μg/kg daily) While the uterine renin before oestradiol administration was significantly below the values observed in mature animals (Table 2) the oviduct renin was in the lower field of the values from mature animals The effect of oestradiol was

TABLE 2 *Renin in Oviduct and Uterus of Immature, Mature and Pregnant Animals*

Animals	Oviduct		Uterus		n
	Mean	Range	Mean	Range	
Immature	*0 044*	0 008–0 106	*0 181*	0 012– 0 397	3
Immature + oestradiol	*0 444*	0 247–0 884	*0 964*	0 477– 1 292	3
Mature	*0 097*	0 026–0 200	*0 728*	0 344– 1 904	8
Pregnant 28 day	*0 109*	0 058–0 185	*6 275*	2 984–10 33	4

The renin concentration is expressed in GU/g In the immature animals the renin concentration of oviduct and uterus was investigated before and after treatment with oestradiol monobenzoate, 10 µg/kg daily in 4 days

an obvious elevation of the uterine renin to values of the mature state, and of the renin of the oviduct to concentrations about 4 times higher than those of mature animals

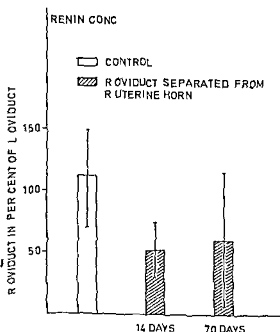


Fig 3 The renin concentration of the right oviduct expressed in per cent of the concentration of the left in unoperated control animals (white column, n = 20 mean 112 + 42 per cent) and in animals exposed to a separation of the oviduct from uterus on the *right side* (hatched columns) for a period of 14 days (n = 7 mean 52 + 22 per cent) and 70 days (n = 7 mean 60 + 55 per cent)

In 4 animals 28 days pregnant the renin concentration of the uterus was about 10 times the level of the non pregnant state, while the renin concentration of the oviduct was almost unchanged

3 *Renin in Oviduct Separated from the Uterine Horn*

Comparing the renin concentration of the right and the left oviduct in 6 normal animals (Group I), it was observed that the values of 20 pairs of identically located biopsies dispersed greatly Expressing the renin concentration of the right oviduct in per cent of the concentration of the left one (Fig 3), the values, nevertheless, did not differ significantly from 100 per cent (mean 112 ± 42 per cent, significance level $0 2 < p < 0 4$)

In 14 animals (Group III) the connexion between the uterus and oviduct, including the mesometrium and the mesoalpinx with the vascular and lymphatic connexions was cut on the right side in order to see whether the renin content of the oviduct depends on the anatomical relation *to the* uterus A 14 day separation of the oviduct from the uterus on the right side in 7 mature animals caused a significant decrease, the mean renin concentration of this oviduct being 52 ± 22 per cent of the mean concentration of the left, untouched oviduct (significance level p =

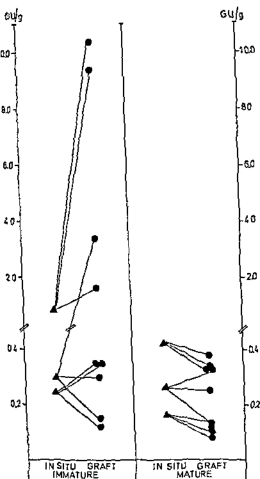


Fig 4 The renin concentration of the oviduct *in situ* (▲) compared with that of intraocular grafts (●) To the left the values of 3 grafts from each of 3 immature animals treated by oestradiol monobenzoate (10 μg/kg daily) for the last 4 days To the right the values of 3 mature animals hysterectomized at the time of the transplantation The lines connect the corresponding values of tissue *in situ* and grafts

0 002) In the remaining 7 animals of the group the operations was performed at the immature stage and the effect investigated about 70 days later The renin concentration of the separated oviduct was here about 60 per cent (mean) of the concentration of the untouched oviduct, the values markedly dispersing (S D ± 55 per cent), but still significantly lower than the control at the 5 per cent level (p = 0 025) The separation of the oviduct from the uterine horn thus resulted in a decreased mean renin content, but never in a disappearance of the renin from the oviduct

4 *Renin in Intraocular Grafts of Oviduct*

In a further attempt to decide whether the oviduct is able to synthesize renin 3 small tissue pieces from the one oviduct were autotransplanted to the anterior chamber of the left eye in each of 6 animals (Group IV) Three of the animals were immature, but treated with oestradiol (oestradiol monobenzoate, 10 μg/kg daily) for the last 4 days of an 18 day period In 5 transplants the values, 0 1–0 4 GU/g, were about identical with the level of the concentration *in situ* (0 25–0 30–0 88 GU/g), but in 4 cases the renin concentration had increased to values, 1 6–10 0 GU/g even higher than usually measured in the normal uterus The renin concentration of 3 of these grafts was about 11 times as high as the concentration of the oviduct *in situ* from the same animal

Intra ocular autotransplantation was further performed on 3 mature animals, which were hysterectomized at the time of the transplantation After 20 days the renin concentrations of the grafts were 0 1–0 4 GU/g (Fig 4) In proportion to the renin concentration of the oviduct *in situ* (0 16–0 42 GU/g) the concentration of the grafts was about 80 per cent (mean 78 ± 25 per cent), not significantly different from 100 per cent (p = 0 10) The results obtained in these hysterectomized animals demonstrated that the renin content of the oviduct is independent of the uterine renin

5 *Histological Examination of the Oviduct*

The histological picture of the oviduct, a muscular tube containing an irregular folded mucous membrane, is shown in Fig 5, demonstrating the dimensional differences in the mucous membrane and the muscular coat in the isthmus (Fig 5A) and the ampulla (Fig 5B)

For transplantation especially the isthmic segment of the oviduct was used and in

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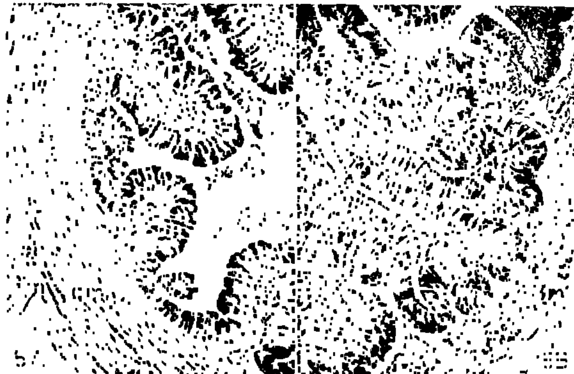


Fig 5 A The isthmus of the oviduct from a normal, mature rabbit The mucous membrane, forming low irregular folds, is covered by a high, columnar epithelium, consisting mainly of secretory, granula containing cells *(se)* The muscular layer is well developed (100–200 micron thick) dominated by circular running muscle cells *(cm)*, only few longitudinal directed bundles *(lm)* peripherally Renin concentration of this segment 0 247 GU/g PAS stain 125 ×

B The ampulla of the oviduct, characterized by a labyrinth of branched folds of mucous membrane covered by a ciliary epithelium *(ce)* containing only few secretory cells The circular muscular coat *(cm)* is rather thin (50–75 micron) Renin concentration of this segment 0 019 GU/g *PAS stain* 125 ×

agreement with this the grafts were characterized (Fig 6) partly by fields of coarsely folded mucous membrane covered by a strongly PAS-positive secreting epithelium, and partly by large bundles of smooth muscle cells, dominated more or less by collagen fibres and fibroblasts Eosinophile granulated cells, a common finding in the uterine endometrium of oestradiol treated animals, but never found in the oviduct *in situ*, were often observed subepithelially in the grafts Apart from these cells and the secreting epithelial cells, any granulated cells were not observed either in the grafts or in the oviduct *in situ*, with special attention turned to the vessel walls In an attempt to correlate the renin concentration of the grafts with the morphological picture it was observed that in the case of a great content of collagen fibres, cells with pycnotic nuclei, and faintly PAS-stained epithelium of the mucous membrane, the renin concentration was always below the concentration of the tissue *in situ* The main part of the grafts, however, showed a well-preserved cell morphology, vascularization and secreting activity of the epithelium without any demonstrable parallelism to the renin concentration Comparing the grafts of the immature, oestradiol treated animals (Fig 6A) with those of mature, hysterectomized animals (Fig 6B) it was obvious that in spite of the great differences in renin concentration the histological picture showed no qualitative or quantitative differences



Fig 6 A Intra-ocular graft from oestradiol treated, immature rabbit Renin concentration of this graft 10 45 GU/g

B Graft from mature hysterectomized animal Renin concentration of this graft 0 102 GU/g Both of the grafts are covered by a high columnar, secretory epithelium and contain scattered bundles of smooth muscle cells increased amounts of connective tissue, and plenty of smaller arterioles and venoles PAS stain 125 ×

DISCUSSION

These experiments show that renin is located to all parts of the genital tract of female rabbits It is remarkable that in addition to the uterus, considerable amounts of renin (0 026–0 200 GU/g) are measured in the oviduct with especially high concentrations (0 127–0 790 GU/g) in the isthmic segment The oviduct renin increases markedly following oestradiol treatment, whereas maturity and terminal pregnancy does not stimulate the renin concentration, contrary to the effect on uterine renin During separation, performed surgically, of the oviduct from the uterine horn on the one side the renin concentration of the oviduct decreases, but probably not more than explained by the impairment of the vascularization caused by the operation By way of intra-ocular tissue transplantation it is finally shown that the oviduct contains renin producing cells, as previously demonstrated for the endometrium and the myometrium of the rabbit uterus (*Eskildsen* 1971) In spite of the lower concentrations (5–20 × 10^{-3} GU/g) in the vagina, mesometrium and ovary an occurrence of renin forming cells is also possible here In comparison with these values the renin concentration of plasma from normal rabbits is 50–100 times lower (about 1 × 10^{-4} GU/ml) Neither in oviduct nor in uterus has it been possible to demonstrate, by help of PAS-stain, any granulated, epithelioid cells, like those described in the juxtaglomerular apparatus of the kidney (*Hartroft et al* 1964 *Faarup* 1968 *Cook* 1971)

The physiological role of renin in the oviduct as well as in the uterus, is so far quite ambiguous, but in view of the known ability of angiotensin to contract smooth muscles, influence sodium transport and interfere with the adrenal steroid release, it is possible that similar functions may take place in the female genital tract It is known that oestradiol treatment, which results in increased renin content of the oviduct, also stimulates the muscular activity (*De Mattos & Coutinho* 1971) and the active secretion (

1956), but the correlation, if any, between these different effects of the treatment is so far unknown

This work was supported by grants from *Nordisk Insulinfond* and the *Legacy of Miss P A Brandt*

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Acta path microbiol scand Section A 80, 751–755, 1972

FOAM CELLS IN THE PLACENTA IN EXTREME HYPERLIPAEMIA

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A case of delivery complicated by extreme hyperlipaemia, probably of the Friederickson type IV, is reported We found numerous foam cells in the placenta, mainly concentrated at the edge of the fibrin deposits in the intervillous space In our opinion, based on the results of light microscopical, histo chemical and electron microscopical examinations, the foam cells are free macrophages originating from the maternal circulation No other essential histological changes were found and the placenta functioned adequately

In cases of hyperlipaemia, foam cells are frequently found in liver, spleen, bone marrow and lymph nodes (9) They have not previously been described as being present in the placenta in connection with hyperlipaemia, although it is not surprising to find them there The reason why they have not been observed in nine previously reported cases of labour complicated by hyperlipaemia (4, 5, 8, 10, 11, 12, 17) is presumably that the placenta was not examined histologically At least, histological examinations are not mentioned in any of the above reports

During labour we found foam cells in the intervillous space of the placenta in a patient with extreme hyperlipaemia

CASE REPORT (14)

The patient was a 25 year-old primigravida without any family history of hyperlipaemia, diabetes mellitus or cardiovascular diseases She had always enjoyed good health apart from two mild transitory episodes of colicky pain in the epigastric region, occurring nine months before the beginning of the pregnancy and during the eighth month of pregnancy

The pregnancy was uncomplicated The patient was checked by her own doctor and in the obstetric out patient clinic, normal conditions were revealed at all examinations

Three days before the calculated term, colicky pain occurred in the epigastric region radiating into the small of the back There was no vomiting, jaundice, fever or discoloration of faeces and urine She was admitted 24 hours later because the pain persisted Her general condition was unaffected, temperature, pulse and blood pressure were normal The abdomen was soft, with diffuse tenderness in the epigastric region, there was no resistance, abnormal swelling or dullness Obstetrical examination revealed normal conditions corresponding to pregnancy at term

A survey X ray of the abdomen showed no signs of pneumoperitoneum or concrements The abdominal pain subsided spontaneously and had disappeared completely 36 hours after it started

Venous blood samples drawn at admission showed heavily lipaemic plasma, se triglycerides were determined at 5700 mg/100 ml and se cholesterol at 1320 mg/100 ml During the first 24 hours there was slight glucosuria and slightly increased urinary diastase (200–600 Somogyi units/100 ml), but blood sugar and serum amylase were normal

Labour was induced 19 hours after admission and, 10 hours later following uncomplicated labour, the patient delivered a boy Birth weight 3650 g,

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length 53 cm The child was completely normal and the extent of bleeding at delivery was normal although the blood was remarkably pink The puerperium was uncomplicated and the patient was discharged 11 days after delivery During the puerperium and follow up over a five month period after delivery, the lipid concentration in the blood decreased gradually Three months after delivery, the serum cholesterol was normal whereas serum triglycerides remained slightly elevated (about 300 mg/100 ml) throughout the entire follow up period

Macroscopical Examination (1)

The placenta weighed 650 g and was circular in shape, 20 cm in diameter The thickness was uniform, 3 cm The maternal surface was normal with cotyledons of normal appearance The umbilical cord was inserted centrally on the foetal surface, and the veins branched off normally There were no thrombi in the veins The umbilical cord was 62 cm long and without knots or discoloration The membranes were normal Upon cutting of the placenta into slices of 1 cm, the cut surface was seen to be normal without focal changes Fixation of the placenta was done in 4 per cent formalin It was observed that, after 24 hours, the fixation liquid was milky

Microscopical Examination

Apart from the foam cells described below, light microscopy reveals

The umbilical cord contains three vessels, one vein and two arteries, presenting normal endothelium in Wharton's jelly of normal appearance There are no foam cells in the umbilical cord The slightly thickened but otherwise normal chorionic plate is covered by a pseudo-stratified amniotic epithelium Langhans' fibrin layer is of normal thickness The size, stroma density and vascularity of the villi are normal The number of syncytial knots corresponds to that normally found at term, whereas the number of cytotrophoblast cells is slightly increased as compared with the normal findings at this time There are no fibrous villi and the quantity of intervillous fibrin is normal There is an increased amount of fibrinoid necroses and, in one single spot, a small intervillous haemorrhage is observed The walls of the vessels in the stem villi are thickened and the endothelium is markedly ballooned The lumen is narrowed The basal fibrin layers are of normal thickness and the decidua stains normally with silver and Toluidine-blue (13)

We found foam cells in all 16 sections The majority lie in clumps consisting of up to 20 cells, but there are also cells lying singly The foam cells are situated in the intervillous space, lying typically at the edge of and partly embedded in the fibrin layers They are present in both Langhans', Rohr's and Nitabuch's fibrin layers, but most frequently at the junction of the last mentioned layer and the intervillous space It was not possible to demonstrate them definitely in the decidua, but they were found in the trophoblast layer and at the junction between the two basal fibrin layers (Fig 1) Furthermore they are seen, although not frequently, in the fibrin deposits facing the stem villi They are not found in the villi but in a few cases, near the syncytium in the terminal villi The cells measure about 35 μm, they are round or polygonal, presenting a distinct cell membrane with an abundant, light, finely granulated cytoplasm The cells have a small chromatin rich nucleus, often centrally situated (Fig 2)

Multinuclear cells are not observed

Sex Chromatin

As the child was a boy, it would have been possible, by counting sex chromatin using Barr's method, to reveal the origin of the foam cells We made this examination but, because of the incommensurability of the various cell types, the result was not sufficiently convincing On the other hand we were able to prove that only about 5 per cent of the foam cells contained a Y chromatin This examination was made by means of fluorescence microscopy of sections stained with 0 5 per cent quinacrine hydrochloride (15)

Histochemical Examination

With PAS staining, the cytoplasm of the foam cells is negative After Lendrum staining, the nuclei are dark and the cytoplasm is finely granulated and bluish With Sudan Black B, the cytoplasm is homogeneously black and there is sporadic staining of the adjoining fibrin layer (Fig 3) Oil red staining produces a red colour of the cytoplasm of the foam cells and drop shaped red spots in the fibrin layers Weaker staining is obtained with Sudan III Nile blue produces no staining and there is no double refraction in polarized light Because of the formalin fixation, the lipoid stainings are not optimum and a definite conclusion as to the nature of the lipoids cannot be drawn

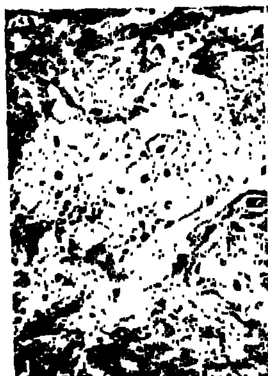
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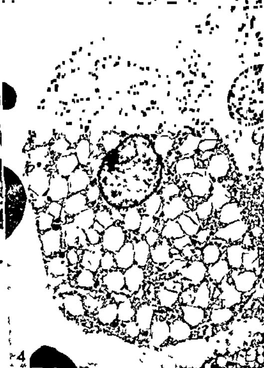
Fig 1 Foam cells in the edge of the fibrin layers. Haematoxylin eosin × 750

Fig 2 Foam cells in 1 μm section Haematoxylin eosin × 1400

Fig 3 Foam cells Frozen sections of formalin fixed tissue Sudan Black B × 400

Fig 4 Electronmicrograph of a foamcell Magnification ca 3000




3

4

However, they are consistent with the supposition that it is triglyceride and non crystalline cholesterol

Electron Microscopy

Because of the fixation in formalin which is far from optimum as regards electron microscopy, the description must be viewed with certain reservation

The foam cell has a rather pyknotic nucleus The cytoplasm contains dense and large vacuoles (Fig 4)

The cell resembles to a certain extent a macrophage There is no structural likeness between the foam cells and cytotrophoblast cells Hofbauer cells or decidua cells (2) Under the electron microscope, the decidua and syncytial cells appear normal The nuclei of the foam cells resemble somewhat the nuclei of the syncytial cells whereas the diameter of the vacuoles in the cytoplasm of the foam cells is about three times that of the vacuoles of the syncytium (3)

DISCUSSION

The pattern of hyperlipaemia, defined as the interrelationship between the triglycerides and the cholesterol and the changes in these values during the course of events, was fundamentally equal to that seen in healthy individuals during pregnancy and puerperium, although the elevations were excessive (18) According to the laboratory investigations there was no pancreatitis and since no other conditions were found which might give rise to a secondary hyperlipaemia, as judged on the basis of the lipoprotein electrophoresis pattern and the course of the puerperium, we supposed that it was an essential hyperlipaemia type IV, as described by *Frederickson* (7) The obliterating endarteritis found in the arteries of the stem villi does not help to clarify the aetiology of the hyperlipaemia because, according to *Fox* (6), it can be found in otherwise normal placentae and also in connection with a series of illnesses in pregnant women Many investigators (3, 19) have described the transformation of Hofbauer cells into foam cells Bearing in mind that several authors consider the Hofbauer cells to be phagocytic (20), foam cells might be assumed to derive from the labile forms of these cells However, it is generally agreed that the cells are of foetal origin and are situated intravillously We found, however, that cells lay exclusively in the intervillous space In conjunction with the morphological picture and the Y chromatin determination, this finding strongly supports the supposition that the foam cells are derived from free macrophages in the maternal circulation

Unfortunately, the child s blood was not examined at birth However, no foam cells were found in the vessels of the villi or the umbilical cord, and this argues against the presumption that the child suffered from hyperlipaemia It should be pointed out that the placenta must have functioned adequately, evaluated on the basis of the weight and histology of the placenta and the weight appearance and growth of the child

The presence of foam cells in the maternal part of the placental circulation in this patient is best explained by the theory that free macrophages from the maternal circulation are transformed into foam cells by phagocytose Because blood smear from the mother is not available we do not know whether there were any circulating foam cells The fact that they accumulate at the foeto-maternal barrier might, in our opinion be explained by the intensive lipoid metabolism in the placenta (16) which increases and decreases the concentrations of the individual lipoid fractions manifold, and which will give rise to local excessive increases in concentration in the intervillous space

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Acta path microbiol scand Section A 80, 756–768, 1972

HISTOLOGY OF THE PROSTATE IN ELDERLY MEN

A Study in an Autopsy Series

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As part of a comprehensive study of the testes, the pituitary gland and the adrenal glands in relation to abnormal growth of the prostate, the occurrence of benign nodular hyperplasia (BNH), carcinoma (C), diffuse atrophy (DA) and normal histology (N) of the prostate was studied in a consecutive series of 206 men over 40 years of age submitted to necropsy Atypical glands without obvious invasive properties were recorded separately and termed atypical glandular proliferation (AGP) The occurrence of BNH and C were both strongly related to age BNH was the most frequent histological pattern encountered (80 1 per cent), and after the age of 70 years, BNH was observed in practically all cases C was observed in 70 patients (34 per cent) and was an incidental finding at autopsy in all but four cases After the age of 80 years, 52 per cent carried C The great majority of carcinomas were small and were located predominantly in the peripheral zone of the prostate The frequency of C in patients previously operated upon for BNH was similar to that in non-operated patients The frequency of BNH in prostates with C was not different from that to be expected as a purely coincidental occurrence The location of AGP resembled that observed for C However, the simultaneous occurrence of AGP and BNH was more frequent than that to be expected as pure coincidence Whether AGP represents benign or potentially malignant lesions remains obscure The classification of AGP as a benign lesion or carcinoma may markedly influence the prevalence ratios of latent prostatic carcinoma in autopsy series In patients dying from cardiovascular disease, BNH occurred more frequently, and C less frequently than what would be expected

Benign hyperplasia and carcinoma of the prostate are increasingly frequent with advancing age and are uncommon before the age of 40 (*Moore* 1935, 1942, *Franks* 1954 a, b) Autopsy studies have revealed benign hyperplasia to occur in more than 95 per cent, and carcinoma of the prostate in approximately 40 per cent, of men over 75 years of age (*Liavåg* 1967 *Lundberg* & *Berge* 1970) The recorded incidence of prostatic carcinoma is rising in Norway, and carcinoma of the prostate is now the most frequent form of cancer among Norwegian men (*The Cancer Registry of Norway* 1969)

The aetiology of benign nodular hyperplasia and carcinoma of the prostate in man is unknown The concept has been advanced that altered function of the testes, the pituitary and the adrenal glands in the ageing male may be causally involved in the development of these conditions (*Lower* 1933, *Huggins* 1947, *Moore* 1947, *Sommers* 1957,

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Stern et al 1964) Furthermore, benign prostatic hyperplasia occurs more frequently in men with diabetes mellitus (*Bourke & Griffin* 1966), whereas a lowered occurrence of prostatic carcinoma in patients with liver cirrhosis has been reported (*Robson* 1966) However, the results of previous studies to detect abnormal function and structure of endocrine glands in patients with prostatic neoplasia or hyperplasia are inconclusive

This report forms part of a comprehensive autopsy study which has been performed to investigate the association between the histological appearance of the prostate and morphological changes in the testes, the pituitary gland and the adrenal glands, and presents the histological findings in the prostates of men over 40 years

MATERIAL AND METHODS

During a 3 months period 1967–68 the prostates from 207 consecutive autopsies of men over 40 years of age were collected One specimen was excluded due to extensive tumour invasion from a bladder carcinoma The study, therefore, is based on 206 cases

The autopsies were usually performed 8 to 32 hours after death. Tissues adjacent to the prostatic capsule were removed before fixation The specimens were fixed in 10 per cent neutral formaldehyde solution for 24 hours and subsequently in 4 per cent neutral formaldehyde solution for 9 days After fixation the prostate was sectioned in 4–6 mm thick slices perpendicular to the prostatic urethra, starting from the cranial end Following dehydration and paraffin embedding one total section was cut from the cranial level of every second slice, and labelled I II respectively Depending on the size of the prostate three to six sections were obtained from each specimen The sections were stained with Harris haematoxylin and eosin orange G acid fuchsin

Histological examination of the prostate was performed by the authors and a third observer* independently and without any knowledge of the clinical data and the diagnosis settled by commission When a conclusive diagnosis could not be reached a fourth observer§ was consulted and the diagnosis settled according to the prevailing opinion

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§ Leif Jorgensen M D

The following histological [illegible] were recorded

1 *Normal histology* (N), i.e. [illegible] atrophic or proliferative changes
2 *Diffuse atrophy* (DA) i.e. [illegible] diffuse atrophic changes of glandular epithelium and stroma without evidence of hyperplasia or neoplasia.
3 *Benign nodular hyperplasia* (BNH), i.e. prostate showing nodular hyperplasia of glandular and stromal components in accordance with the description given by *Moore* (1943)
4 *Carcinoma* (C), the criteria being atypical glandular pattern, cellular atypism and invasion of perineural spaces or blood vessels. When invasion of fibromuscular stroma was clearly evident, it was accepted as a criterion of malignancy These criteria are similar to those described by *Moore* (1935), *Dixon & Moore* (1952), *Franks* (1954 b) and *Lee* [illegible] (1957)
5 *Atypical glandular proliferation* (AGP), i.e. prostate showing foci of atypical glands with slight or moderate cellular atypism, but without conclusive evidence of invasive growth (Fig 1)

The presence of atypical glandular proliferation in prostates showing carcinoma was not recorded. When either atypical glandular proliferation or carcinoma occurred in glands showing benign nodular hyperplasia both conditions were recorded

The outlines of all carcinomas and foci of atypical glandular proliferation were drawn on the microscopical slide under low power magnification Every section showing either of these lesions were magnified or reduced to a transverse diameter of 4 cm in a Liesegang RAJ\H III S projector The outlines of the projected [illegible] of the section with its foci of carcinoma or atypical glandular proliferation and urethra, were drawn to present the location and extent of these lesions (Figs 3 5) In addition the outlines of carcinoma and atypical glandular proliferation drawn on the slides were transferred by free hand onto standardized diagrams showing transverse sections of the prostate The centre of each lesion from all cases was plotted onto a common diagram for each level The standard diagram was divided into two geometrically equal zones a central and a peripheral The lesions were recorded as either central or peripheral according to the location of their centres

Clinical data Informations about the age of the patients previous operations on the prostate carcinoma of the prostate diagnosed before death previous treatment with oestrogenic hormone and clinically manifest diabetes mellitus were recorded from the clinical notes. The duration of the final illness after onset of symptoms was classified as a) less than 1 day b) 1 7 days and c) more than 7 days Based on the autopsy report the underlying cause of death was settled and for [illegible]



Fig 1 Focus of atypical glandular proliferation Harris' haematoxylin and eosin orange G acid fuchsin stain × 63

reasons, grouped into three categories cardio vascular disease (including cerebrovascular and peripheral vascular disease)†, malignant tumour, other conditions

In all cases, sections from the liver were examin ed histologically for the presence of liver cirrhosis

Statistical methods Expected numbers of patients with a particular condition within sub groups of the material were calculated from the age-specific ratios of this condition in the total mate rial

The number of cases in which two lesions could be expected to occur in the same gland was calculated from the formula $F_1 \times F_2 \times T$, F_1 and F_2 being age specific prevalence ratios of the lesions in the total material and T the total number of patients in the corresponding age group The age specific prevalence ratios of atypical glandular proliferation were calculated from the total number of glands not showing carcinoma

In the comparison of observed and expected numbers, Cochran's X^2-test (*Armitage* 1971) with one degree of freedom was performed P values below 0 05 were considered statistically significant

† Includes death from myocardial infarction (54 cases), cerebrovascular and peripheral vascular disease (22 + 9 cases), rheumatic valvular disease (4 cases), miscellaneous cardiovascular disorders (14 cases)

″ In this group, death was caused by trauma or intoxication (13 cases), chronic lung disease (12 cases), senile dementia (8 cases), chronic renal disease (4 cases), miscellaneous (10 cases)

RESULTS

Table 1 presents the total distribution of histological diagnoses of the prostates in 206 patients by age Benign nodular hyperplasia was the most frequent finding, and was observed in 80 1 per cent (165/206) of the patients This figure includes glands in which benign nodular hyperplasia occurred together with carcinoma or atypical glandular proliferation Glands showing benign nodular hyperplasia alone accounted for 40 8 per cent (84/206) of all glands examined The total occurrence of benign nodular hyperplasia increased with age, reaching a maximum of 95 5 per cent (64/67) in the eighth decade (Fig 2)

TABLE 1 *Histological Diagnoses of the Prostates by Age*

Age	No patients	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	6	1	1	4	0	0	0	0
50–59	38	11	5	12	3	1	6	0
60–69	66	7	3	25	18	6	6	1
70–79	67	0	1	33	26	1	5	1
80+	29	0	1	10	14	1	3	0
Total	206	19	11	84	61	9	20	2

N = normal prostate DA – diffuse atrophy, BNH = benign nodular hyperplasia, C – carcinoma AGP = atypical glandular proliferation

Carcinoma of the prostate was observed in 70 patients (34 0 per cent), all being over 50 years of age Among the patients with prostatic carcinoma, four (5 7 per cent) had the malignancy diagnosed before death and had received oestrogen treatment Among 202 patients in whom prostatic malignancy was not clinically suspected, carcinoma was revealed at autopsy in 32 7 per cent The relative frequency increased with age, and 52 per cent of patients more than 80 years of age carried a prostatic malignancy (Fig 2)

Figures 3 and 4 show the location and extent of carcinomas at different levels of the prostate Only non operated patients in whom the carcinoma was incidentally detected at autopsy (55 cases) are included The tumours were generally small and preferably located in the peripheral zone of the prostate In the apical region (levels III IV), carcinomas were not strictly peripherally located In the more cranial parts of the gland (levels I-II), carcinomatous foci were less common anterior to the urethra Carcinomatous foci were more numerous in

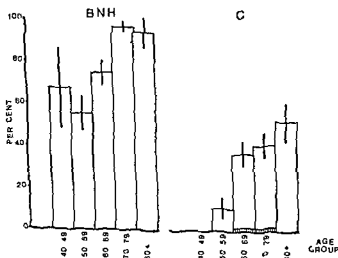


Fig 2 Prevalence ratio (± standard error of the percentage) of benign nodular hyperplasia (BNH) and carcinoma (C) of the prostate in 206 consecutive autopsies Hatched parts of columns denote clinically manifest carcinoma

TABLE 2 *Relationship between Carcinoma (C) and Benign Nodular Hyperplasia (BNH) of the Prostate Observed and Expected Frequency of Their Combined Occurrence*

		With BNH	
Age	No	Observed no	Expected no
Patients with C			
40–49	0	0	0
50–59	4	3	2 2
60–69	24	18	17 8
70–79	27	26	25 8
80 +	15	14	14 0
Total	70	61	59 8
Patients without C			
Total	136	104	105 2

sections from the apical region in spite of the generally smaller size of these sections, than in those from the middle and upper parts of the prostate Cancer foci totalled 132 among which 105 (80 per cent) had their centres in the peripheral zone, as defined in

TABLE 3 *Relationship between Atypical Glandular Proliferation (AGP) and Benign Nodular Hyperplasia (BNH) of the Prostate Observed and Expected§ Frequency of Their Combined Occurrence*

		With BNH	
Age	No	Observed no	Expected no
Patients with AGP			
40–49	0	0	0
50–59	6	6	3 2
60–69	7	6	5 2
70 79	6	5	5 7
80 +	3	3	2 8
Total	22	20	16 9
Patients without AGP			
Total	114	75	78 1

§ Calculations based on age-specific prevalence ratios of AGP and BNH in prostates not showing carcinoma

TABLE 4 *Prostatic Carcinoma (C) in Relation to Previous Prostatic Surgery for Benign Nodular Hyperplasia*

		With C	
Age	No	Observed no	Expected no
Operated patients			
40–49	0	0	0
50–59	3	0	0 3
60–69	5	1	1 8
70–79	7	4	2 8
80 +	9	6	4 7
Total	24	11	9 6
Non operated patients			
Total	182	59	60 4

this study This figure is significantly higher than that to be expected on the basis of geometric considerations ($X^2 = 23\,047$, $p < 0\,0005$)

The occurrence of atypical glandular proliferation in patients without prostatic carcinoma remained fairly constant (15–21 per cent) with advancing age These lesions were small, occasionally multifocal and mostly peripherally located (Fig 5) Their location resembled that observed for carcinomas, but the difference in the number of foci with a central (12) or a peripheral (24) location was not statistically significant ($X^2 = 2\,000$, $p > 0\,10$)

A histologically normal prostate was encountered in 19 patients (9 2 per cent), all of whom were under 70 years of age Diffuse atrophy of the prostate was observed at all ages (Table 1) and was in all cases associated with debilitating diseases of long duration

Among patients with carcinoma of the prostate, the majority (61/70) also had benign nodular hyperplasia The frequency of carcinoma accompanied by benign nodular hyperplasia is close to what would be expected to occur from pure coincidence, as calculated from the age-specific prevalence rates for each lesion (Table 2)

Only two patients had atypical glandular

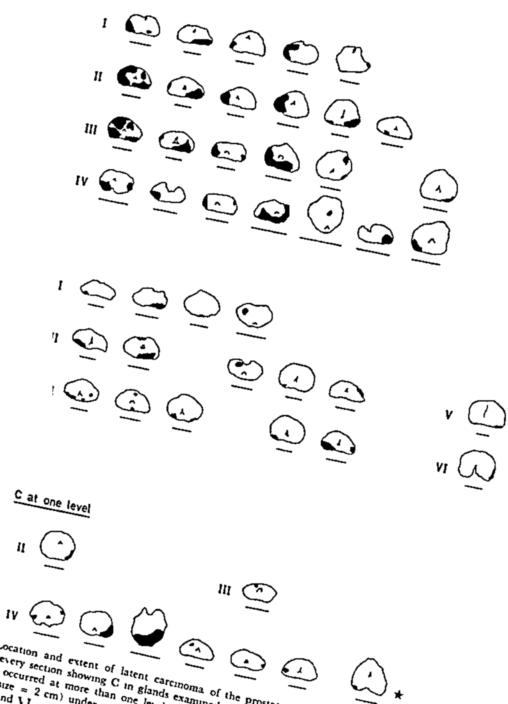
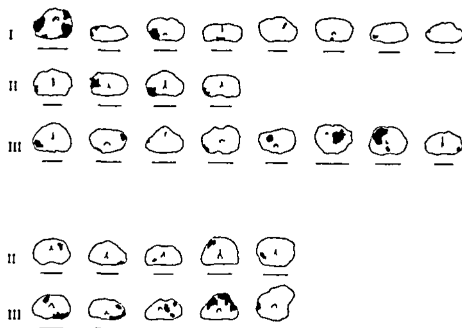


Fig 3 Location and extent of latent carcinoma of the prostate (C) in 23 non-operated cases Projections of every section showing C in glands examined at four levels (I–IV) Projections from glands in which C occurred at more than one level are presented in vertical rows Scale is indicated by line (natural size = 2 cm) under each section V–VI One case examined at six levels and showing C at levels V and VI
* One case examined at five levels and showing C at level IV

C at more than one level

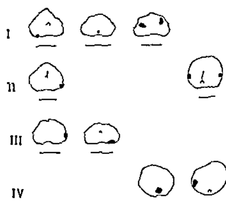


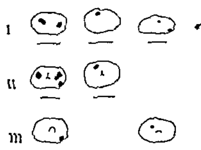
C at one level



Fig 4 Location and extent of latent carcinoma of the prostate (C) in 32 non-operated cases Projections of every section showing C in glands examined at three levels (I–III) Projections from glands in which C occurred at more than one level are presented in vertical rows Scale is indicated by line (natural size = 2 cm) under each section

AGP at more than one level





AGP at one level

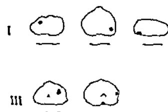




Fig 5 Location and extent of atypical glandular proliferation of the prostate (AGP) in 20 non operated cases *Projections of every section showing AGP in glands examined A) at four levels (I–IV) and B) at three levels (I–III)* Projections from glands in which AGP occurred at more than one level are presented in vertical rows Scale is indicated by line (natural size ≈ 2 cm) under each section

TABLE 5 *Histological Diagnosis of the Prostate§ in Patients with Diabetes Mellitus and Liver Cirrhosis Observed (O)* and Expected (E) Numbers*

	No patients	Histological diagnosis							
		N		BNH		C		AGP	
		O	E	O	E	O	E	O	E
Diabetes mellitus	9	1	0 4	8	7 9	2	3 6	1	1 6
Liver cirrhosis	5	2	0 5	2	3 0	1	1 2	1	0 8

§ For abbreviations, see Table 1
* Exceeds total number of patients since C and AGP were accompanied by BNH

49*

TABLE 6 *Prostatic Carcinoma (C) in Patients Carrying Non Prostatic Malignant Tumours*

Patients with non prostatic tumour		With C	
		Observed	Expected
Primary site	No	no	no
Stomach	12	4	4 2
Colon	3	1	1 0
Respiratory tract	18	7	5 1
Urinary tract	7	2	2 0
Haematopoietic and lymphatic system	10	4	3 0
Other	12	5	4 5
Total	62	23	19 8
Patients without non prostatic tumour			
Total	144	47	50 2

proliferation which was not accompanied by benign nodular hyperplasia The frequency of the combination of these lesions was significantly higher than that to be expected to occur from pure coincidence ($X^2 = 6\ 759$, $p < 0\ 01$) (Table 3)

The occurrence of prostatic carcinoma in 24 patients previously operated on for benign prostatic hyperplasia appears from Table 4 Sections from the surgical specimens were available in 18 cases and, on re examination, the diagnosis of benign hyperplasia only was confirmed in all cases The number of patients in whom carcinoma was observed did not differ substantially from that of patients in whom it would be expected to occur according to the age-specific prevalence ratios in the total material Statistically, the difference was not significant ($X^2 = 0\ 440$, $p > 0\ 50$)

Nine patients had clinically manifest diabetes mellitus which had lasted for 2–22 years, on the average 8 years, and five patients had liver cirrhosis In neither category was the distribution of histological diagnoses significantly different from that to be expected from the distribution in the total material (Table 5) One patient, 58 years of age, who had been treated with insulin for 22 years, showed a histologically normal prostate

Table 6 presents the occurrence of prostatic carcinoma in patients carrying non prostatic malignant tumours In the group as a whole, prostatic carcinoma occurred with a frequency which was slightly higher than that to be expected However, this frequency did not differ significantly from that among patients without non prostatic malignant tumours ($X^2 = 1\ 165$, $p > 0\ 20$) Neither seemed prostatic carcinoma to be related to non prostatic malignancies at any particular site

Patients dying from cardiovascular disease demonstrated a frequency of benign nodular hyperplasia higher than that to be expected and a frequency of carcinoma lower than that to be expected (Table 7) The frequencies differed significantly from those in patients dying from other causes, including malignant tumours ($X^2 = 5\ 281$ and $X^2 = 6\ 021$ respectively, $p < 0\ 025$)

COMMENT

This investigation has confirmed that benign nodular hyperplasia is the most frequent growth pattern encountered in the prostate of elderly men This lesion is frequently observed in the fifth decade, the occurrence increases with age and after the age of 70

TABLE 7 *Occurrence of Benign Nodular Hyperplasia (BNH) and Carcinoma (C) of the Prostate in Patients Dying from Cardiovascular Disease Observed (O) and Expected (E) Numbers*

Cause of death	No patients	With BNH		With C	
		O	E	O	E
Cardiovascular disease	103	90	84 1	29	36 8
Malignant tumour and other conditions	103	75	81 0	41	33 1

years, practically all men have benign nodular hyperplasia of the prostate This is in agreement with previous reports (*Moore* 1942, *Franks* 1954 a, *Liavåg* 1967), and a histologically normal prostate must be expected to be exceptional in old men

The high frequency (34,0 per cent) of prostatic carcinoma in the present series of men over 40 years of age is in agreement with other autopsy studies reported from Austria (*Moore* 1935), Denmark (*Starklint* 1950), Great Britain (*Franks* 1954 b), U S A (*Butler et al* 1959), Norway (*Liavåg* 1967) and Sweden (*Lundberg* & *Berge* 1970) The occurrence of prostatic carcinoma increases with age, and in this series about 50 per cent of men over 80 years of age carried a prostatic carcinoma The great majority of carcinomas were incidental findings at autopsy, and correspond to those which have been called *unsuspected carcinoma* (*Muir* 1934, *Liavåg* 1967), *early carcinoma* (*Moore* 1935), or *latent carcinoma* (*Andrews* 1949, *Starklint* 1950, *Franks* 1954 b)

Direct comparison of total rates of prostatic carcinoma in different series may be misleading In comparable series from Scandinavia, the frequency of prostatic carcinoma incidentally detected at autopsy appears to be more common in Sweden (Table 8) However, when age differences are adjusted for, the frequencies do not differ significantly ($X^2 = 5\,073$, d f $= 2$, $p > 0\,05$)

There is a great discrepancy between the high frequency of prostatic carcinoma revealed at autopsy and the occurrence of carcinoma which has been clinically suspected (clinically manifest carcinoma) or death from prostatic carcinoma Whether latent carcinoma found at autopsy represents a lesion biologically different from the clinically manifest carcinoma has been a matter of dispute In clinically manifest carcinoma the tumour is generally larger than in latent carcinoma, but the histological features are essentially the same (*Starklint* 1950, *Liavåg* 1967) The fact that the majority of prostatic carcinomas incidentally detected at autopsy are small, well differentiated, and do not extend beyond the confines of the prostate, indicates that these tumours have a low growth rate On the other hand, approximately 30 per cent of patients with prostatic carcinoma unexpectedly detected at operation for benign hyperplasia die from prostatic malignancy (*Bauer et al* 1960, *Miller* & *Seljelid* 1971) This is a substantially higher percentage than that to be expected from the death rates of prostatic carcinoma otherwise seen in consecutive autopsy series These patients, therefore, represent a selected group from which no conclusions as to the biological potential of the carcinoma incidentally detected at autopsy should be drawn

Most authors claim that prostatic carcinoma is preferably located in the peripheral regions of the gland, as appears both from studies of carcinoma detected at autopsy (*Franks* 1954 b) and in glands obtained by radical perineal prostatectomy (*Blennerhassett* & *Vickery* 1966) In the present series, the majority of carcinomatous lesions were

TABLE 8 *Prevalence Ratios of Prostatic Carcinoma (C) Incidentally Found at Autopsy in Three Series from Scandinavia*

	Total no patients	No with C	Prevalence ratios	
			Crude	Age adjusted
Liavåg (1967)*	330	90	27 3	28 7
Lundberg & Berge (1970)§	292	116	39 7	37 1
Present series*	202	66	32 7	33 4

* Norway
§ Sweden

small and predominantly located in the peripheral parts of the prostate Without assuming an equal growth rate in all directions from the site of origin, the suggestion that carcinoma of the prostate arises preferably in a peripheral zone of the gland may therefore be justified Whether the predominance of carcinomatous foci in this region is due to a greater gland density near the capsule (*McNeal* 1969) or to other biological differences between central and peripheral parts of the prostate cannot be answered on the basis of the present material

It is generally held that there is no causal relationship between nodular hyperplasia of the prostate and carcinoma (*Dixon & Moore* 1952), although some investigators have claimed that the two conditions are associated more often than if chance alone was responsible (*Andrews* 1949, *Edwards et al* 1953) In the present series however, the frequency of benign hyperplasia in prostates with carcinoma was not different from that to be expected to occur from pure coincidence This together with the fact that carcinoma occurred in glands without benign hyperplasia, and the zonal difference in the occurrence of benign hyperplasia and carcinoma (*Scott* 1963) indicate that different pathogenetic factors are involved in the development of the two conditions Concerning the pathogenesis and the predominantly central location of benign hyperplasia, recent studies by *Siiteri & Wilson* (1970) showing a higher accumulation of dihydrotestosterone in the periurethral than in the outer regions of the gland may be of significance

In patients subjected to prostatic surgery a lowered frequency of latent carcinoma has been reported (*Liavåg* 1967) No such difference has been observed in the present series This we attribute mainly to the fact that the peripheral parts of the gland, in which carcinoma is most likely to occur, are left behind at operation for benign prostatic hypertrophy

In this study, atypical glands without obvious invasive properties were frequently encountered in the prostate at all ages These *atypical glandular proliferations,* which probably correspond to the lesions termed "doubtful carcinomas" by *Franks* (1954 b), might possibly have been interpreted as true carcinomas by other investigators Classification of such lesions as carcinomas would increase the frequency of latent carcinoma in this series by 10 to 15 per cent (Fig 6) Whether such atypical focal proliferations represent benign or potentially malignant lesions remains obscure Observations in surgical material have indicated that similar lesions may develop into carcinomas (*Miller & Seljelid* 1971) In our series, foci of atypical glandular proliferation were slightly more frequent in the peripheral zone, thus resembling the distribution of carcinomatous foci On the other hand, their occurrence together with benign hyperplasia was more frequent than that to be expected if the two lesions were not associated (Table 3)

Diabetes mellitus has been suggested as one factor in the pathogenesis of benign prostatic hyperplasia (*Roberts* 1967), and a high incidence of diabetes in patients with benign hyperplasia has been reported (*Bourke & Griffin* 1966, 1968) Whether carcinoma of the prostate is more common in diabetics is disputed (*Bell* 1957, *Joslin et al* 1959, *Lea*

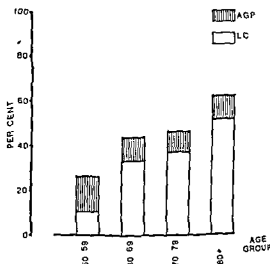


Fig 6 Combined prevalence ratio of latent carcinoma (LC) and atypical glandular proliferation (AGP) of the prostate in 206 consecutive autopsies.

1966), and fasting blood sugar levels in subjects with prostatic carcinoma were similar to those in controls (*Wynder et al* 1971). Among the small number of patients with diabetes mellitus in this series, the frequencies of benign nodular hyperplasia and carcinoma were not significantly different from the frequencies of the total material.

Retrospective studies have demonstrated a lowered frequency of prostatic carcinoma in men with severe liver cirrhosis, whereas the occurrence of benign hyperplasia in cirrhotics was similar to that in non cirrhotics (*Glantz* 1964, *Robson* 1966). The occurrence of prostatic carcinoma and hyperplasia in the present series did not differ significantly from the frequencies in the total material, but the small number of patients with liver cirrhosis does not allow definite conclusions.

An association between cardiovascular disease and prostatic enlargement was claimed by Zinsser (1969). In patients dying from cardiovascular disease, including cerebrovascular and peripheral vascular diseases, in this series, the observed frequency of benign prostatic hyperplasia was greater than expected. This observation also coincides with a previous report according to which hypertension is more common in patients suffering from benign hyperplasia of the prostate (*Bourke* & *Griffin* 1966). It was suggested that prostatic hypertrophy is a manifestation of relative excess of oestrogen secretion and that the increased blood pressure was related to the salt- and water retaining properties of oestrogens. Prostatic carcinoma, on the other hand, was less common than expected among patients dying from cardiovascular disease in the present series, the reasons for which are obscure.

In papers to follow, the relationship between the histological findings in the prostate and prostatic weight, and weights and morphological characters of the testes, the pituitary gland and the adrenal glands of the patients presented here, will be analysed.

We are indebted to Professors *Kristen Arnesen* M D and *Leif Jorgensen*, M D, Department of Pathology, Ullevål Hospital, for participating in the histological examination.

The technical assistance of Mrs *I J Ingebrigtsen* is gratefully acknowledged.

This study was financially supported by a grant from *The Norwegian Society for Fighting Cancer*.

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Acta path microbiol scand Section A 80, 769-777, 1972

PROSTATIC WEIGHT IN ELDERLY MEN

An Analysis in an Autopsy Series

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Prostatic weight was analysed in a consecutive autopsy series of 172 men over 40 years of age among whom none had clinically manifest carcinoma of the prostate or had previously been subjected to prostatic surgery The weight of the prostate increased with age only in glands showing benign nodular hyperplasia (BNH) Carcinoma often occurred in small glands (less than 20 grams), and carcinomatous glands generally had lower weight than glands with BNH alone Prostatic weight showed a positive and significant correlation to body weight Patients dying from cardiovascular disease and patients with clinically manifest diabetes mellitus had higher prostatic weight than others Multiple regression analysis showed a significant correlation between prostatic weight and age On the other hand a strong correlation between age and BNH existed Thus, when age was not included in the analysis, it was shown that of the factors tested in the present series the presence of BNH body weight, death from cardiovascular disease, and diabetes mellitus were of significance for prostatic weight variability, although the total effect of these factors was relatively low It may be advisable to correct for these factors in analyses of prostatic weight in an autopsy material

The hormone dependency of prostatic function and growth has been well documented by observations in animals Castration is followed by profound atrophic changes of the prostate, but can be prevented by the administration of androgenic hormones (*Scott* 1953)

In experimental procedures, prostatic weight is frequently used to assess the effect of endocrine stimulation upon accessory sex glands In man, studies of prostatic weight have been concerned mainly with its relation to age and glandular histology A marked weight increase has been observed in glands with benign hyperplasia and carcinoma (*Simmonds 1918, Roessle* & *Roulet* 1932, *Liavåg* 1967)

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We intended to study prostatic weight in relation to prostatic histology and the weight and morphological characters of the testes the pituitary gland and the adrenal glands in an attempt to uncover endocrine factors related to prostatic growth However, in man several factors may interfere with prostatic weight and disturb its usefulness as a measure of endocrine stimulation The present report deals with the association between prostatic weight and prostatic histology, age, body weight, body length, treatment with steroid hormones, diabetes mellitus, liver cirrhosis, cause of death and the duration of final illness The effects of these factors have been assessed by single variable analysis and by multiple regression analysis which facilitates a simultaneous study of several variables and a ranking of their relative importance

This report forms part of a comprehensive

autopsy study which has been performed to investigate the association between the histological appearance of the prostate and morphological changes in the testes, the pituitary gland and the adrenal glands The histological findings in the prostate in this series have been previously reported (*Harbitz & Haugen* 1972)

MATERIAL AND METHODS

Prostatic glands were collected from 207 consecutive autopsies of men over 40 years of age Thirty patients subjected to prostatic surgery or oestrogen treatment, four patients with secondary tumour invasion of the prostate or testis, and one patient with seminoma of the testis were not included in the main analysis which thus is based on 172 cases

Weight and Histological Classification of the Prostate

The weight of the unfixed prostate was recorded after removal of extracapsular tissue The techniques of fixation, microscopical examination of total transverse sections, and histological classification have been described previously (*Harbitz & Haugen* 1972)

The presence of normal prostatic histology (N), benign nodular hyperplasia (BNH), carcinoma (C) atypical glandular proliferation (AGP) or diffuse atrophy (DA) was noted for each gland The occurrence of atypical glandular proliferation was not specified in glands where carcinoma was diagnosed

Clinical Data

Clinical data were recorded from the clinical notes and prepared for electronic computer analysis

Statistical Methods

Differences between means were tested by a modified Student s t test accounting for unequal variances and numbers of groups (*Snedecor & Cochran* 1967) P values below 0 05 were regarded statistically significant

Adjustment for age differences were performed according to the indirect method of standardization (*Armitage* 1971), using the age specific mean prostatic weights of the main material of 172 prostates as standard weights

Multiple regression analysis Forward stepwise regression analysis (*Draper & Smith* 1966) was applied using prostatic weight (X_1) as the dependent variable The following factors either bivariate (1,0) (labelled X_2 to X_{12}), or continuous (labelled X_{13} to X_{15}), were included in the analysis as *explanatory* (independent) variables

Histology of the Prostate

X_2 Benign nodular hyperplasia (BNH)
X_3 Atypical glandular proliferation (AGP)
X_4 Carcinoma (C)
X_5 Diffuse atrophy (DA)

Cause of Death

X_6 Cardiovascular disease§
X_7 Malignant tumour

Duration of Final Illness

X_8 1–7 days
X_9 > 7 days

Other

X_{10} Steroid hormone treatment*
X_{11} Diabetes mellitus
X_{12} Liver cirrhosis
X_{13} Age
X_{14} Body weight
X_{15} Body length

As to the bivariate variables, the presence of the characteristic in question gives the variable the value 1, whereas the absence of this characteristic gives the variable the value 0 Normal histology of the prostate is included by coding 0 for all histology groups listed Causes of death other than cardiovascular disease and malignant neoplasm and duration of final illness less than 1 day are all coded in a similar way

A linear regression of X_1 on X_2 to X_{15} was assumed, and the multiple regression equation $X_1 = b_0 + b_2X_2 + b_3X_3 + \quad\quad + b_{15}X_{15}$ was employed, b_0 being a constant and b_n the regression coefficient for X_1 on X_n

Forward stepwise regression analysis was run until all explanatory variables which were partially significant at the 5 per cent level at each step, were included

The analysis was based on a standard program for multiple regression (NRSR) developed at the Norwegian Computing Center, Blindern Oslo Norway and was conducted on a Univac 1108 computer

§ includes death from myocardial infarction (49 cases), cerebrovascular and peripheral vascular disease (16+8 cases), rheumatic valvular disease (4 cases) miscellaneous cardiovascular disorders (11 cases)

* includes treatment with corticosteroids (7 cases), anabolic steroids (nortestosterone) (5 cases), or both (11 cases)

TABLE 1 *Histological Diagnoses of the Prostate by Age in 172 Patients*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	1	1	2	0	0	0	0
50–59	11	5	10	3	1	5	0
60–69	7	1	22	15	5	6	1
70–79	0	1	28	21	0	5	1
80+	0	0	9	9	0	2	0
Total	19	8	71	48	6	18	2

N = normal histology, DA = diffuse atrophy, BNH = benign nodular hyperplasia, C = carcinoma AGP = atypical glandular proliferation

TABLE 2 *Prostatic Weight (Grams) by Age*

Age	No patients	Mean	SD
40–49	4	19 5	5 4
50–59	35	22 1	5 2
60–69	57	26 4	10 6
70–79	56	29 3	17 3
80+	20	42 6	33 2
All	172	28 2	17 2

SD Standard deviation

RESULTS

The histological findings in the prostates of the 172 patients included in the main analysis are presented in Table 1

Prostatic weight varied from 10 3 to 155 4 grams, the median being 23 8 grams The mean prostatic weight increased with advancing age (Table 2) The difference between the total mean prostatic weight (28 2 grams) and the median indicates a skewness in the distribution which also appears from Figure 1

In glands showing normal histology (Fig 2 a) or diffuse atrophy (Fig 2 b), the range of prostatic weight was less than in glands showing atypical glandular proliferation (Fig 2 c), benign hyperplasia only (Fig 2 d) or carcinoma (Fig 2 e) Among glands showing benign nodular hyperplasia alone, 11 3 per cent (8/71) weighed less than 20 grams, whereas 31 5 per cent (17/54) of prostates with carcinoma had such low weight

The lowest age-specific mean weights were seen in patients with normal histology (N), diffuse atrophy (DA) or carcinoma (C) and atypical glandular proliferation (AGP) without benign hyperplasia (Table 3) The highest age specific mean weights were mostly observed in patients in whom benign nodular hyperplasia occurred alone (BNH) Glands with carcinoma accompanied by benign hyperplasia (C + BNH) had lower mean weights, although the difference between the two groups was statistically significant in the seventh decade only ($p < 0.05$)

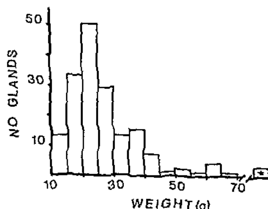
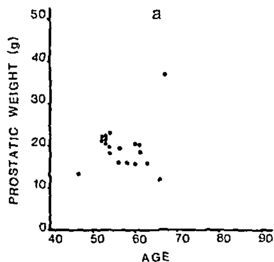
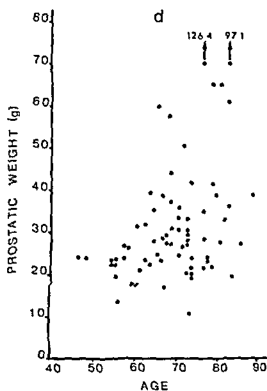
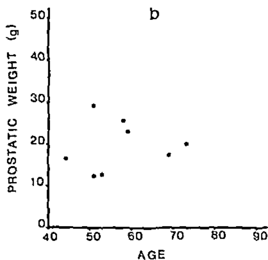
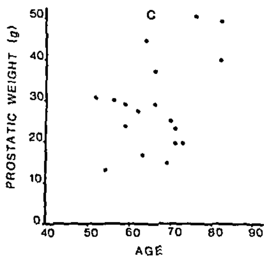


Fig 1 Frequency distribution of prostatic weight at autopsy in 172 men previously not subjected to prostatic surgery or oestrogen treatment * Three glands weighing 97 1, 126 4 and 155 4 grams


a
PROSTATIC WEIGHT (g)
50
40
30
20
10
0
40
50
60
70
80
90
AGE


d
126.4
97.1
PROSTATIC WEIGHT (g)
80
70
60
50
40
30
20
10
0
40
50
60
70
80
90
AGE


b
PROSTATIC WEIGHT (g)
50
40
30
20
10
0
40
50
60
70
80
90
AGE


c
PROSTATIC WEIGHT (g)
50
40
30
20
10
0
40
50
60
70
80
90
AGE

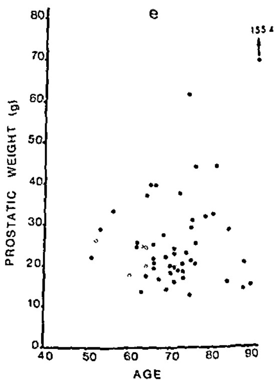

e
155.4
PROSTATIC WEIGHT (g)
80
70
60
50
40
30
20
10
0
40
50
60
70
80
90
AGE

TABLE 3 *Mean Prostatic Weight§ (Grams) by Histological Diagnoses of the Prostate* and Age*

Age	N	DA	BNH	C + BNH	C	AGP + BNH	AGP
40–49	(13 4)	(16 7)	24 0	–	–	–	–
50–59	20 1	20 8	21 9	28 1	(26 4)	23 8	–
60–69	20 0	(17 5)	31 7	24 4	19 4	28 5	(15 9)
70–79	–	(20 1)	33 3	25 5	–	27 7	(13 5)
80 +	–	–	45 2	39 6	–	44 7	–
All	19 7	19 8	32 5	28 0	20 6	28 8	14 7
S D	5 2	6 1	18 0	21 1	6 0	11 5	1 7

§ Figures in brackets refer to single observations * For abbreviations, see Table 1
S D Standard deviation

TABLE 4 *Prostatic Weight (Grams) in Relation to Cause of Death, Duration of Final Illness, Steroid Hormone Treatment, Diabetes Mellitus and Liver Cirrhosis*

	No patients	Observed Mean	Observed S D	Age adjusted mean
Cause of death				
Cardiovascular disease	88	32 5	21 4	32 1
Malignant tumour	44	22 1	7 1	23 1
Other	40	25 5	11 5	24 9
Duration of final illness				
< 1 day	37	29 6	14 6	30 4
1–7 days	35	30 3	20 9	32 6
> 7 days	100	27 0	16 8	26 0
Steroid hormone treatment	23	21 6	7 4	22 5
Diabetes mellitus	8	40 8	38 2	34 9
Liver cirrhosis	5	16 9	4 2	18 8

S D Standard deviation

In glands without benign hyperplasia, prostatic weigth was not related to age, and was similar to that of glands with normal histology The mean weight of glands with benign hyperplasia either alone (BNH) or together with atypical glandular proliferation (AGP + BNH) increased with age On the other hand, no definite relationship to age was demonstrable in carcinomatous glands The high mean weight among the latter in the highest age group is strongly influenced by one extreme observation (Table 3 and Fig 2)

Fig 2 a–e Prostatic weight plotted against age in patients with normal histology (a), diffuse atrophy (b) atypical glandular proliferation with (●) or without (O) benign nodular hyperplasia (c), benign nodular hyperplasia only (d), and carcinoma with (●) or without (O) benign nodular hyperplasia (e) of the prostate

Table 4 presents the observed and age-adjusted mean prostatic weight in relation to cause of death, duration of final illness, steroid hormone treatment, diabetes mellitus, and liver cirrhosis Prostatic weight in patients

TABLE 5 *Relationship between Prostatic Weight and Various Explanatory Variables Expressed by Correlation Coefficients Simple Correlation Analysis*

Explanatory variable	X_1 Prostatic weight ($n_1 = 172$)	Significant at level
Histology of the prostate §		
X_2 BNH ($n = 137$)	0 254	0 001
X_3 AGP ($n = 20$)	–0 018	0 814
X_4 C ($n = 54$)	–0 041	0 593
X_5 DA ($n = 8$)	–0 108	0 157
Cause of death		
X_6 Cardiovascular disease ($n = 88$)	0 254	0 001
X_7 Malignant tumour ($n = 44$)	–0 209	0 006
Duration of final illness		
X_8 1–7 days ($n = 35$)	0 061	0 426
X_9 > 7 days ($n = 100$)	–0 085	0 268
Other		
X_{10} Steroid hormone treatment ($n = 23$)	–0 133	0 082
X_{11} Diabetes mellitus ($n = 8$)	0 162	0 034
X_{12} Liver cirrhosis ($n = 5$)	0 114	0 136
X_{13} Age ($n = 172$)	0 352	< 0 001
X_{14} Body weight ($n = 172$)	0 206	0 007
X_{15} Body length ($n = 172$)	–0 002	0 978

§ For abbreviations, see Table 1

n_1 Number of cases in which prostatic weight was recorded

n Number of cases in which the characteristic in question was either present (for bivariate variables) or recorded (for continous variables)

dying from cardiovascular disease was significantly higher than that in patients dying from malignant tumours ($p < 0\ 001$) or other causes ($p < 0\ 025$) The mean prostatic weight in patients whose final illness had lasted for more than one week was apparently reduced Patients who had received treatment with steroid hormones had low prostatic weight Diabetics demonstrated high mean prostatic weight, but the variability of the observations was wide Five patients with liver cirrhosis showed low prostatic weight

As a preparatory procedure, simple correlation analysis was performed prior to the multivariate analysis, and the relationship, expressed by correlation coefficients, between prostatic weight and the individual explanatory variables (X_2 to X_{15}) appears from Table 5 A positive and significant correlation between prostatic weight and benign nodular hyperplasia was demonstrated The correlations to other histological diagnoses were negligible and not significant Among the continous variables (X_{13} to X_{15}), a significant and positive correlation for prostatic weight to age and body weight was demonstrated Among the remaining variables (X_2 to X_{12}), prostatic weight was positively correlated to cardiovascular disease (X_6) and diabetes mellitus (X_{11}) Prostatic weight was negatively correlated to malignant tumour (X_7), protracted final illness (X_9), steroid hormone treatment (X_{10}) and liver cirrhosis (X_{12}), but only the relationship to malignant tumour as a cause of death was statistically significant

Obviously some of the factors mentioned above are positively correlated with each other The majority of patients dying from malignant tumours were chronically ill and had lower body weight (mean 56 7 kg) than patients dying from cardiovascular diesase

(mean 67 1 kg) or other diseases (mean 63 3 kg) Steroid hormone treatment had preferably been given to patients in this category Furthermore, most patients with diabetes mellitus were old (mean 74 1 years), carried benign nodular hyperplasia of the prostate, and died from cardiovascular disease The observed correlation between prostatic weight and age (Table 5) was apparently linked to an interrelation between age and the occurrence of benign nodular hyperplasia of the prostate (*Harbitz & Haugen* 1972) since it could be demonstrated that prostatic weight would increase with age only in cases of benign nodular hyperplasia (Table 3)

Multiple Regression Analysis

The strong correlation between BNH (X) and age (X_{13}) was confirmed by a separate regression analysis (partial correlation coefficient = 0 411, $p < 0\ 001$) Initially, multiple regression analysis (stepwise procedure) was performed with age as one of the explanatory variables Age was selected at the first step causing the greatest reduction in the variance of prostatic weight In addition, cardiovascular disease as a cause of death and body weight were included at the chosen level of significance (Table 6 a) The explanatory value of this set of variables (expressed as R^2) was 0 221 By this procedure, prostatic weight did not show a significant correlation to any of the histological groups However, if age were not included in the analysis (Table 6 b), benign nodular hyperplasia caused the greatest reduction in the variance of prostatic weight Again, a positive correlation between prostatic weight and cardiovascular disease and body weight was demonstrated In addition, diabetes mellitus was now selected at the chosen level of significance

If age be disregarded, prostatic weight can be predicted from the regression equation as

$$X_1 = 1\ 054 + 10\ 059X_2 + 5\ 316X_6 + 13\ 413X_{11} + 0\ 250X_{14}$$

This implies that prostatic weight in this series would increase on the average by about 10 grams in the presence of benign nodular hyperplasia, by 0 250 grams per kilogram

TABLE 6 *Correlation between Prostatic Weight and Regressors Selected by Stepwise Procedure of Multiple Regression Analysis*

	X_1 *Prostatic weight* (n_1 = *172*)		
	Partial correlation coefficient	Partial regression coefficient	Significant at level
a *With age as explanatory variable*			
X_{13} Age (n = 172)	0 383	0 660	<0 001
X_{14} Body weight (n – 172)	0 234	0 272	0 002
X_6 Cardiovascular disease (n – 88)	0 183	5 874	0 017
Multiple correlation coefficient (R)	0 470		<0 001
b *Without age as explanatory variable*			
X_2 BNH§ (n = 137)	0 247	10 059	0 001
X_{14} Body weight (n = 172)	0 209	0 250	0 007
X_{11} Diabetes mellitus (n = 8)	0 174	13 413	0 024
X_6 Cardiovascular disease (n 88)	0 159	5 316	0 039
Multiple correlation coefficient (R)	0 410		<0 001

§ Benign nodular hyperplasia
n_1 and n For explanation see Table 5

body weight by about 5 3 grams if death is due to cardiovascular disease, and by about 13 grams in cases with clinically manifest diabetes mellitus However the standard deviations of the regression coefficients b_6 (cardiovascular disease) and b_{11} (diabetes mellitus) were high The explanatory value of the selected set of variables (expressed as R) was low (0 168) This means that only 16 8 per cent of the variation in prostatic weight was explained by these factors

COMMENT

As far as we are informed prostatic weight as a measure of endocrine stimulation of the prostate has hitherto not been explored in man For this purpose patients presenting normal histology of the prostate early stages of carcinoma or moderate hypertrophy appear more suitable than patients with advanced stages of pathological growth By exclusion of patients previously subjected to prostatic surgery or oestrogen treatment we assume that the remainder comprise earlier stages of prostatic carcinoma and hyperplasia The carcinomas in the present series were generally small (*Harbitz* & *Haugen* 1972) and the weight increase of glands with benign hyperplasia was generally moderate

The weight increase with advancing age in glands with benign nodular hyperplasia implies that this process is more extensive in high age In agreement with previous observations by *Simmonds* (1918) and *Teem* (1936) glands with normal histology showed no significant weight age relationship and the observed increase of prostatic weight with age was obviously linked to the presence of benign hyperplasia

In the present series glands with carcinoma had lower mean weight than those with benign nodular hyperplasia which is in contrast to previous observations on latent prostatic carcinoma by *Hirst* & *Bergman* (1954) and *Liavåg* (1967) Carcinoma occurred frequently (17/46) in glands weighing less than 20 grams and the weight of carcinomatous glands not showing benign hyperplasia did not differ from that of normal glands This coincides with the fact that the great majority of carcinomas in this series were small and a noticeable weight increase would therefore be unexpected Benign nodular hyperplasia is present in the majority of glands with carcinoma (*Liavåg* 1967 *Harbitz* & *Haugen* 1972) and increased weight in glands with latent carcinoma as compared to normal glands is therefore mainly due to the presence of benign hyperplasia Our results do not support the observation made by *Andrews* (1949) and *Liavåg* (1967) that benign hyperplasia is more extensive in glands with latent carcinoma than in non carcinomatous prostates Similarly since the foci of atypical glandular proliferation were also small their contribution to prostatic weight is negligible This is consistent with the result of the multiple regression analysis in which benign hyperplasia was the only histological pattern significantly correlated with prostatic weight

Prostatic weight showed a positive and statistically significant correlation to body weight both at simple and multiple regression analysis The low prostatic weight in patients dying from malignant tumours in patients with protracted final illness or treated with steroid hormones other than oestrogens is explained by the fact that these factors are intercorrelated with each other and with body weight The observations of a low glandular weight in these patients is in accordance with previous findings of atrophic changes of the prostate in debilitating disease (*McNeal* 1968) Since no correlation between prostatic weight and body length could be demonstrated on the other hand prostatic weight seems to depend rather upon the nutritional state than upon body size *per se*

The multiple regression analysis revealed a positive relationship between prostatic weight and cardiovascular disease as a cause of death This association can not wholly be attributed to the more frequent occurrence of benign hyperplasia (*Harbitz* & *Haugen* 1972) or the higher body weight in these patients since cardiovascular disease was

positively correlated to prostatic weight even when these factors were accounted for (Table 6b). Similarly, a positive correlation between prostatic weight and diabetes mellitus could be demonstrated even when the influence of benign hyperplasia, body weight and cardiovascular disease as a cause of death was accounted for. Prostatic weight in patients with diabetes mellitus remained higher than that in any other group even when their high age was adjusted for (Table 4). However, the high standard deviation of prostatic weight in diabetics weakens the significance of this observation. Our results do not support the clinical impression of subnormal size of the prostate in diabetics as reported by *Schoffling et al.* (1963). Although the correlations observed in the present series were weak, they may imply additional factors leading to increased prostatic weight in patients suffering from cardiovascular disease or diabetes mellitus. In these patients, therefore, benign hyperplasia appears both more frequent (*Bourke & Griffin* 1966, *Harbitz & Haugen* 1972) and may be more excessive.

Although the number of cirrhotics in the present series was small, the observations are in consistency with an inhibitory effect of liver cirrhosis on prostatic enlargement as suggested by *Stumpf & Wilens* (1963).

The explanatory value of statistically significant factors at multiple regression analysis whether with (22.1 per cent) or without (16.8 per cent) age as an explanatory variable was low. This may be due to failing linearity of the regression or that important unknown factors related to prostatic weight have not been included in the analysis. However, since prostatic weight was significantly related to benign nodular hyperplasia, body weight, cardiovascular disease and diabetes mellitus, these factors must be considered in further analysis of prostatic weight variability in autopsy materials.

We are indebted to Professors *Helge Stalsberg*, M.D. and *Knut Westlund*, M.D. for helpful advice on the statistical analysis and for reading the manuscript. Actuary *Ingar Holme*, Cand. real., The Norwegian Computing Center, Oslo, Norway performed the computer analysis and gave valuable advice.

This study was financially supported by a grant from The Norwegian Society for Fighting Cancer.

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Acta path microbiol scand Section A 80, 778-784, 1972

THE IN VITRO MATURATION OF THE EMBRYONIC CHICKEN THYMUS

1 Development of an Organ Culture System

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An organ culture technique is described which permits the lymphoid development of the thymic anlagen of 10 day-old chick embryos for at least 10 days *in vitro* Grid organ cultures of a modified Trowell type were employed Among several tissue culture media tested, Waymouth's MB 752/1 medium gave superior results In initial experiments this medium was supplemented with heat inactivated horse serum and embryo extract Subsequently it was found that this supplement could be substituted for by chicken serum alone The morphological development of the embryonic thymic anlagen in organ culture has been characterized Typical lymphoid cells developed from the initial lymphoid precursor cells Late in the culture period, large numbers of small lymphocytes appeared Extensive cell proliferation was demonstrated during the first 6 days of culture by means of autoradiography This organ culture technique should facilitate the further analysis of factors influencing thymic lymphocytopoiesis as well as of the role of the thymus in the development of lymphocyte functions

The epithelial anlage of the chicken thymus originates from the third and fourth pharyngeal pouches (5) Blood borne immature lymphoblasts migrate early into this epithelial anlage and presumably constitute the precursors for the later developing thymic lymphocytes (9, 12)

We have initiated a series of investigations designed to directly assess the role of the thymic micro-environment in the morphological and functional differentiation of the lymphocytes from the first lymphoid precursor cells in the thymus

Hence, the organ culture technique described in this communication was developed It permits the development of the embryonic chicken thymus from a stage containing predominantly small numbers of immature lymphoblasts to a more mature organ with large numbers of typical lymphoid cells

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MATERIAL AND METHODS

Chick embryos Fertilized eggs of the outbred White leghorn De Kalb Chix strain 161 (Hinseberg Hatchery Hinseberg Sweden) were stored at + 12° C until incubated The eggs were incubated in an automatic egg incubator (Funki Type 1, Funki Hammerum, Denmark) at 37 5° C and a relative humidity of 55 per cent The eggs were incubated standing on the pointed end and were automatically turned every hour

Procurement of thymic anlagen The thymic anlagen with adjacent jugular veins of 10 day-old chick embryos were dissected out under the stereomicroscope using sharpened watchmakers forceps, and immediately placed in organ culture

Tissue culture medium Waymouth's MB 752/1 (WM) medium (Grand Island Biologicals, Grand Island N Y, U S A) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) was used in most experiments

Ham s Nutrient Mixture F 10 (F 10) (Grand Island Biologicals), Parker s Medium 199 (M 199) and the monolayer modification of Eagle s Minimum Essential Medium (MEM) (State Bacteriological Institute, Stockholm, Sweden) were also tested Culture times of 6 and 10 days were used Several different medium supplements were tested

Supplements Horse serum (HS) was obtained from the State Bacteriological Laboratory, Stockholm, Sweden Chicken serum (CS) was obtained from young chickens, maintained in our own animal quarters The serum concentration in the tissue culture medium was always 10 per cent (v/v) Both untreated and heat inactivated (56° C for 30 minutes) sera were tested

Embryo-extract (EE_{50}) was prepared from 9 day-old embryos homogenized in equal volumes of Dulbecco s phosphate buffered saline The homogenate was centrifugated at 100 000 × g for 1 hour and heat inactivated at 56° C for 30 minutes The concentration of EE_{50} in the medium was always 5 per cent (v/v) Both sera and embryo-extracts were stored at —70° C until use

Organ culture system The organ cultures were of modified Trowell type (6, 10, 11) Plastic, 35×10 mm tissue culture dishes (Falcon Plastics Oxnard Cal., U S A) each containing 2 ml culture medium were used Each dish contained a 60 mesh stainless steel grid on which a Millipore filter pore size 0 45 μ (Millipore Filter Corp, Bedford Mass, U S A.) was placed in the gas-medium interphase The filters were covered with slices of Spongostan gelatin foam (Ferrosan Malmo Sweden) which were allowed to wet One whole thymic anlage was then placed on each grid Cultures were established in at least triplicates. The organ cultures were incubated in gas tight chambers at 37 5° C using a water saturated atmosphere consisting of 5 per cent CO_2 57 per cent O_2 and 38 per cent N_2 Atmospheres consisting of 5 per cent CO_2 in either air or O_2 did not improve the culture results

Culture periods of up to 10 days were tested Half of the medium was replaced with fresh medium at day 5 if the culture time exceeded 6 days

Autoradiographic technique The cell proliferation in the cultured thymic anlagen was assessed in 3 separate experiments at day 0, 2, 4 6 and 9 of culture by a 4 hour incubation with H^3 methyl thymidine (H^3 TdR) specific activity 6 7 c/mM (New England Nuclear Corp, Boston, Mass U S A), using a H^3 TdR concentration of 1 μc/ml medium Prior to this incubation the thymic anlagen were removed, with the Spongostan from the grids and floated freely on the isotope containing medium The thymic anlagen were then fixed in cold 4 per cent carbonate buffered formalin, dehydrated cleared in xylene and embedded in paraffin 5 μ sections were made and autoradiograms prepared by the liquid-emulsion technique (7), using NTB 2 emulsion (Eastman Kodak Co, Rochester, N Y, U S A), for details see (4)

The slides were exposed for 14 days at +4° C under desiccation and subsequently developed in Kodak D 19 B for 2 min and fixed in Kodak F 24 for 10 minutes The sections were stained through the emulsion with Mayer s haemalum

Evaluation of thymic development The morphology of the thymic anlagen was evaluated by light microscopy of coded histological 5 μ sections prepared and stained as described above The degree of lymphoid structure development was scored as follows —, no typical lymphoid cells, ±, few lymphoid cells, +, many lymphoid cells, + +, many typical lymphoid cells and size of the thymic anlagen larger than +

RESULTS

Role of the tissue culture medium As summarized in Table 1, Waymouth's MB 752/1 medium was much superior to the F10, Medium 199 and Minimum Essential Medium in supporting the lymphoid development of the thymic anlagen of the 10 day-

TABLE 1 *The Effect of Different Tissue Culture Media on the Lymphoid Development of Embryonic Thymic Anlagen*

Medium*	Development of lymphoid thymus† Day 6§	Day 10
F10	± to +	±
M199	± to +	± to +
MEM	±	+
WM	+ +	+ +

* Both chicken serum (CS) and CS + embryo-extract (EE_{50}) were tested as medium supplements One separate experiment for each day with cultures in quadruplicates for each treatment For abbreviations see Material and Methods.

† Scored with respect to lymphoid structure development

— = No typical lymphoid cells
± = Few lymphoid cells
+ = Many lymphoid cells
+ + = Many typical lymphoid cells and size of the thymic anlagen larger than +

§ Half of the medium was replaced on the third day of culture

50*

TABLE 2 *Summary of the Tests of Different Tissue Culture Medium Supplements*

Medium composition*	No of experiments	Development of lymphoid thymus†
WM	7	—
WM + HS (inact)	11	—
WM + EE_{50}	4	—
WM + HS + EE_{50}	2	±
WM + HS (inact) + EE_{50}	'1	+ to + +
WM + CS	1	+ +
WM + CS (inact)	5	+ +
WM + CS + EE_{50}	2	+ +
WM + CS (inact) + EE_{50}	5	+ +

* For explanation of the abbreviations, see Material and Methods

† See Table 1 for explanation of the scoring of the histological development Culture periods of 6 days

old chicken embryos This was apparent from culture times of both 6 and 10 days, using medium supplements of either chicken serum (CS) or CS + embryo extract (EE_{50})

Role of tissue culture medium supplements Thymic anlagen of 10-day-old embryos were cultured for 6 days in Waymouth's MB 752/1 tissue culture medium with different supplements as indicated in Table 2 The effects of these on the development of the lymphoid structure and the size of the anlagen were scored after examination of histological sections under the light microscope The results are summarized in Table 2

In initial experiments, the culture medium was supplemented with heat inactivated horse

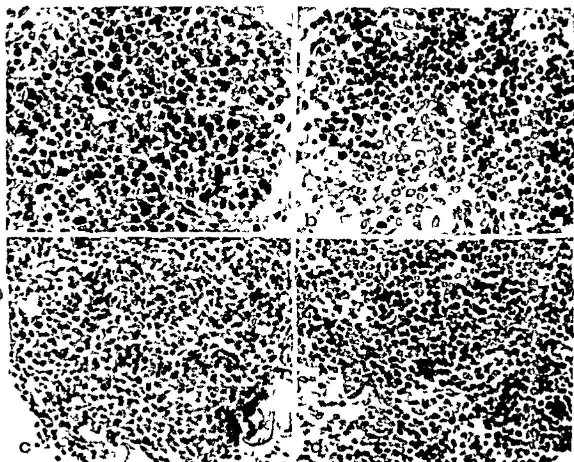


Fig 1 *a* Thymic anlage of 10 day chicken embryo at the initiation of the organ cultures *b* Thymic anlage after 6 days in culture Medium supplement HS + EE_{50} *c* Same as b but HS only as medium supplement *d* Thymus of 16 day embryo (390 ×)

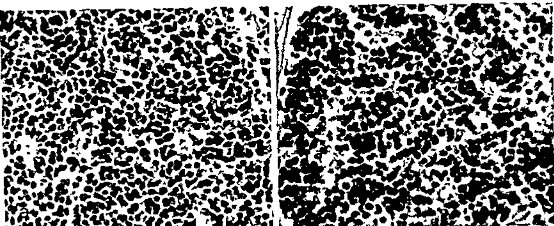


Fig 2 Thymic anlagen of 10 day embryos after 6 days in culture *a* Medium supplement CS + EE_{50} *b* Medium supplement CS only (390 ×)

serum (HS) + embryo extract (EE_{50}) This medium permitted the development of a lymphoid thymus from the undeveloped stage of embryonic day 10 during the 6-day-culture period (Figure 1 a and b) In contrast, the basic medium alone or supplemented only with heat inactivated HS or only with EE_{50} did not support the development of typical lymphoid cells although a few lymphoblasts were present (Figure 1 c) The morphology of the *in situ* developed thymus of a 16 day-embryo is demonstrated for comparison (Figure 1 d)

Heat inactivation of the HS was a necessary step because untreated HS in combination with EE_{50} resulted in poor development of thymus (Table 2) In later experiments, inconsistent results were obtained with heat-inactivated HS and EE_{50} as medium supplement, possibly because not all HS batches supported thymic development equally well Therefore, as indicated in Table 2, heat inactivated or normal chicken serum (CS) alone or in combination with EE_{50} were tested as medium supplements It was found (Table 2) that CS alone as supplement permitted an excellent development of the lymphoid thymus The combination of the CS with EE_{50} did not improve the morphological development (Figures 2 a and b) The morphology of the thymic anlagen of 10 day old embryos after a 6 day period in organ culture corresponded approximately to that of the thymus of the 16-day-old embryo (Figure 1 d)

Cellproliferation in the organ cultured thymus The proliferation of the lymphoid cells in thymus organ cultures established from 10 day old embryos, using a tissue culture medium supplemented with heat-inactivated HS and EE_{50}, was studied after H^3-TdR pulses at day 0, 2, 4, 6 and 9 Figures 3 a-e demonstrate that cells in DNA synthesis were present at all times The number of labelled cells was relatively low at the initiation of the cultures (Figure 3 a), it was high at days 2 6 (Figures 3 b-d) and sharply decreased at the 9th day of culture (Figure 3 e) At day 9, most of the cells were small lymphocytes

Because of the difficulty involved in distinguishing individual lymphoid and epithelial cells, particularly if they were heavily labelled no attempts were made to estimate the proportion of labelled cells of these categories Where identifiable the labelled cells belonged to the lymphoid series and were predominantly large and medium size lymphocytes The finding that the pure epithelial anlagen in the organ cultures containing no supplement in the medium were of the same size as those grown in medium with HS + EE_{50} but contained few labelled cells (results not shown), indicates further that the major-

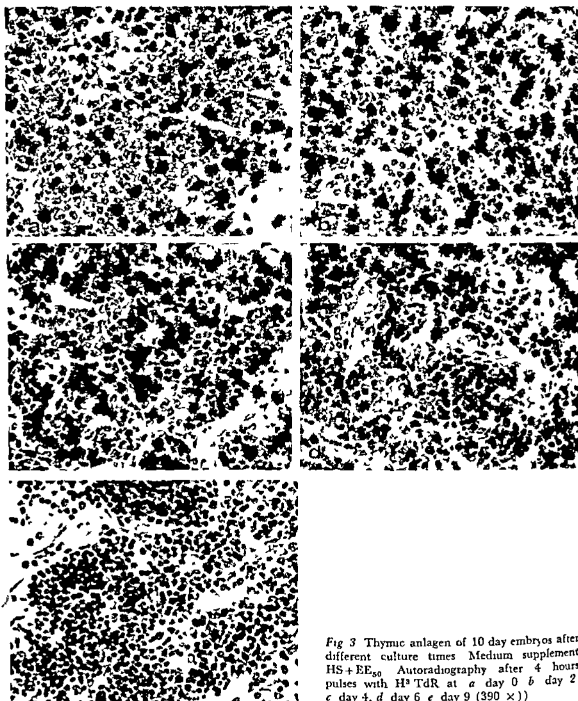


Fig 3 Thymic anlagen of 10 day embryos after different culture times Medium supplement HS + EE_{50} Autoradiography after 4 hours pulses with H^3 TdR at *a* day 0 *b* day 2 *c* day 4, *d* day 6 *e* day 9 (390 ×))

ity of the labelled cells were non epithelial in nature and probably belonged to the lymphoid series

The time-dependent morphological development of the thymus in organ culture In the organ cultures of embryonic thymic anlagen a progressive time dependent development of the thymus was noted This involved an increase of the size of the anlagen, in the number of lymphoid cells as well as a change in the morphological characteristics of the lymphoid cells The increase of the size of

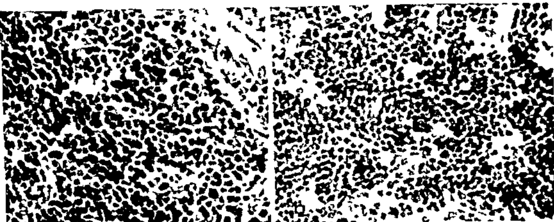


Fig 4 *a* Thymic anlage of 10 day embryo after 10 days in organ culture Medium supplement CS only *b* Thymus of 20 day embryo (390 ×)

the thymus was due both to an increase of the epithelial anlage and an increase in the number of lymphoid cells The thymic anlagen of 10 day old embryos were small and contained relatively small numbers of cells resembling lymphoblasts No small lymphocytes were found at this stage (Figures 1 a and 3 a) The first few small lymphocytes were noted after 2–4 days (Figures 3 b and c) The proportion of small lymphocytes increased progressively and these cells predominated at 10 days of culture (Figure 4 a) At this stage, the lymphoid cell density was comparable to that of the thymus of the 20 day old chick embryo (Figure 4 b), but the frequency of typical small lymphocytes appeared to be lower

DISCUSSION

An organ culture technique permitting the development of the thymic anlagen of 10 day old chick embryos during at least a 10 day period *in vitro* has been described To our knowledge, other adequate organ culture techniques to be used for the embryonic chick thymus do not exist However, *Auerbach et al* succeeded in obtaining lymphoid development in embryonic mouse thymus cultures using a filter well technique (2, 3) Recently the morphological development of the embryonic mouse thymus in this culture system has been further characterized by *Mandel & Russell* (8)

The design of the organ cultures in the present investigation was similar to the Trowell type (6) described by *Nilsson* (10, 11) The most important modifications involved the use of Millipore membranes as support for the Spongostan gelatin foam, the use of Waymouth's MB 752/1 medium with different supplements as will be further discussed, and an atmosphere of 5 per cent CO , 57 per cent O and 38 per cent N_2 Other tissue culture media tested were clearly inferior to the Waymouth's medium

In the initial experiments in the present investigation good lymphoid development was obtained with a tissue culture medium supplemented with heat inactivated horse serum (HS) and embryo extract (EE_{50}) Neither of these alone supported the *in vitro* lymphoid development Furthermore, heat inactivation of the HS was found to be necessary suggesting that this contained one or more heat labile growth inhibitory factors for chick thymus

Somewhat variable results relative to the lymphoid development using HS and EE_{50} as medium supplements were encountered This appeared to be associated with the various HS batches employed For this reason, and also because it seemed desirable to simplify the medium supplement, chicken

serum (CS) with or without EE_{50} was tested It was found that CS in itself sufficed as medium supplement By this means a better reproducibility of the *in vitro* thymic develop ment was also obtained

The main purpose of this investigation was to develop a system in which the role of the thymus in the development of lymphocytes reactive to non specific mitogens such as phytohaemagglutinin (PHA) and concanava lin A (Con A) as well as to histocompatibi lity antigens could be investigated It was therefore an advantage to avoid the use of whole embryo extract which presumably contains a multiplicity of allogeneic histo compatibility antigens

The fact that chicken serum as medium supplement permitted the lymphoid develop ment of the thymus should also greatly facilitate the analysis of the thymic growth promoting factors present in chicken serum In particular it should be possible to directly define the relation between hypophyseal hor mones and thymic development (13) Such studies are in progress

As will be described elsewhere (1) the morphological differentiation of the lympho cytes in the embryonic thymic anlagen in or gan culture is associated with a development of specific functions of these cells Thus we have demonstrated a development of lympho cytes reactive to the mitogens phytohaemag glutinin and concanavalin A The organ culture technique presented may therefore provide an opportunity of studying the role of the thymus in the functional differentia tion of lymphocytes

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Supported by grants from the *Swedish Cancer Society* (proj no 511 B71 01X) and the *Ex pressens prenatalfond*

The skilful technical assistance of Miss *Annsofi Holst* and Miss *Marianne Eriksson* is gratefully acknowledged

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Acta path microbiol scand Section A 80, 785-800 1972

THE NUMBER OF GLOMERULAR CELLS AND SUBSTRUCTURES IN EARLY JUVENILE DIABETES

A Quantitative Electron Microscopic Study

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A quantitative study of glomerular cells of some cell organelles and of basement membrane structures has been performed in control subjects and in patients with juvenile, short term diabetes Cell counts showed that the percentage distribution of the three glomerular cell types, as well as cell density, were normal in the groups of diabetics in which thickening of the peripheral basement membrane and increased amounts of mesangial basement membrane like material had been demonstrated Cellular hyperplasia therefore is not a primary event in the development of diabetic glomerulopathy Cisternae in the endoplasmic reticulum containing basement membrane like material known to be involved in basement membrane synthesis showed a tendency to occur with increased frequency in patients with a few years duration of diabetes Quantitation of heterogeneous inclusion bodies in epithelial cells and of crater like formations on the basement membrane gave similar results Laminated bodies and whorled banded fibers were observed more frequently in diabetic patients This suggests that the basement membrane material in diabetic glomeruli is not entirely normal On the basis of the findings it is tentatively suggested that increased basement membrane synthesis plays a role in the pathogenesis of diabetic glomerulopathy

Diabetic glomerulosclerosis is an integral part of the generalized microangiopathy which almost inevitably develops in the long term diabetic patient

It has been well known for several years that the characteristic feature of diabetic microangiopathy is an augmentation of the basement membrane material (39) The histologic appearance of advanced glomerular lesions in diabetic patients is highly characteristic Increased amounts of basement membrane are found both in the capillary walls and in the mesangial regions In some cases the mesangial basement membrane like material assumes a spherical shape, thus forming the characteristic Kimmelstiel Wilson nodule

In severely affected glomeruli it is obvious that not only the basement membrane but also the cellular components are involved in the pathologic process Mesangial cell hyperplasia as estimated by light microscopy, has been described by many authors (7, 9, 10, 15 ... 18 29 35) and in one study decrease in the number of mesangial cells was reported (6 Some authors have described hyperplasia of endothelial cells (2 32) Decrease in the number of epithelial cells was stressed by Kimmelstiel s group (18 21) whereas

Received 13 vi 72

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Azerad et al (1964) reported epithelial hyperplasia

Hyperplasia of mesangial cells has been described as an early event and was supposed to play a causative role in the development of basement membrane changes (7, 14, 22) All of the above mentioned studies, in only a few of which a quantitative technique was used (16, 18, 21, 35), were hampered by difficulties in distinguishing the three glomerular cell types at the light microscopic level of resolution Statements on the number of glomerular cells which are not based on quantitative studies must be regarded with great reservation

The present study on differential cell counts in patients with short term diabetes and in controls was primarily carried out to test the postulate that the number of mesangial cells is increased initially Discrepancies in previous studies further stimulated the present investigation at the electron microscopic level, which permits identification of the three cell types with a high degree of certainty It was possible to make this study, because electron microscopic photomontages of glomerular cross sections, used for the quantitation of basement membrane material (36, 41) were available

Various cellular and basement membrane structures were also counted on the same glomerular photomontages The cellular organelles encountered are cisternae in the endoplasmic reticulum of epithelial cells containing basement membrane like material, and membrane bound heterogeneous inclusion bodies in epithelial cells

While it is possible to obtain an estimate of the amount of glomerular basement membrane, its fine structure can not as yet be described quantitatively Evaluation of basement membrane structure in the present material concerns therefore only two well defined features lamellated bodies and moon crater-like formations

Rounded lamellated bodies in the glomerular basement membrane material have been described in other diseases (27, 28) and they are frequently observed in glomeruli from long-term diabetics Moon-crater-like formations on the peripheral glomerular basement membrane have been reported from this laboratory in diabetic patients and in rats with experimental diabetes (38) In order to compare the occurrence of these structures in normal non diabetic glomeruli and in glomeruli from patients with early diabetes, they were quantitated on the glomerular photomontages

MATERIAL AND METHODS

The present study was performed on kidney biopsies obtained from young non diabetics and from patients with short term juvenile diabetes The material has been presented in detail previously (41) The diabetic patients are grouped as follows *group I* 7 diabetics at the acute onset of their disease They were biopsied at admission to hospital for newly diagnosed diabetes mellitus before any treatment had been given A second biopsy was obtained about one month later after intensive treatment with insulin and diet *group II* 5 diabetics after a duration of diabetes of 1½–2½ years These patients are identical with 5 of the patients in group I *group III* 8 patients with a duration of diabetes of 3½–5 years Five non diabetic patients without any clinical or laboratory signs of kidney disease served as the control group The ages of the control patients ranged from 12–28 years and of the diabetics from 13 to 34 years

Percutaneous kidney biopsies using the Iversen Roholm needle (8), were obtained in most cases In 4 of the non diabetics, however, a needle biopsy was obtained at laparotomy from the exposed kidney Small blocks of tissue were fixed with OsO_4 dehydrated with acetone and embedded in Vestopal W Thin sections of glomeruli were photographed with a Philips EM 200 after staining with uranyl acetate (34) or double staining with uranyl and lead (15) The total area of the glomerular cross section was photographed, and the electron micrographs produced at a magnification 13 500 × were pasted together, to make a photomontage of the glomerular cross section 84 out of the 103 montages represented glomerular cross sections containing the vascular pole At the vascular pole the glomerulus was delineated with a straight line connecting the two points of reflection of Bowman s capsule onto the capillary tuft

The ' cell count was in reality a count of nuclei Any section of a nucleus large enough to be identified as such was counted as a cell Indented nuclei seen in epithelial cells, may obviously appear on the micrograph as 2 isolated segments. Therefore two neighbouring nuclear seg

ments were counted as two cells only if a cell border could be identified between them All the cell counts were made at least twice

The distinction between the three cell types in the glomerular tuft generally offered no problems The following criteria were used Nuclei inside the capillaries were counted as endothelial cells whether or not the connection with the capillary wall was seen on the section *unless* cytoplasmic characteristics provided evidence that it was a leucocyte Nuclei counted as mesangial cells were those which except for their intraluminal cytoplasmic protrusions were without contact with the capillary lumen in the plane of section They were surrounded on all sides with basement membrane material and/or endothelial cells According to the definition an endothelial cell may occasionally be counted as a mesangial cell

Epithelial cell nuclei were those located in the urinary space, *unless* cytoplasmic characteristic (lysosomes smooth endoplasmic reticulum arrangement of mitochondria etc) showed that it was detached tubular cells

For each cross section the percentage distribution of the three cell types was determined Also the 'density' of cells within each cross section was calculated as the number of cells per 1000 μ^2 The size of the glomerular cross sections was calculated as that of an ellipse measuring the largest diameter inside Bowman s capsule and the largest diameter perpendicular to this (in cm s) The density of mesangial cells was further calculated as the number per 100 μ^2 of mesangial area. This was done in only 56 glomerular cross sections in which the total mesangial area had been measured with planimetry (42)

The cellular organelles which were counted on each of the cross sections, were defined by a number of structural characteristics

A cisterna in the endoplasmic reticulum (ERC) of an epithelial cell containing basement membrane like material is illustrated in Fig 1 Definition of this organelle was based on the presence of a rather homogenous, rounded mass of fibrillar material with the same fine structure as the basement membrane surrounded by a membrane, which was studded with ribosome granules on the cytoplasmic side

The heterogeneous inclusion bodies (HB) in epithelial cells as illustrated in Fig 4 were surrounded

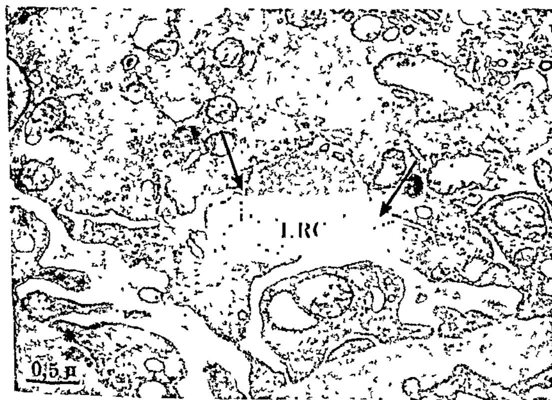


Fig 1 Basement membrane like material in a dilated cistern in the endoplasmic reticulum (ERC) of a glomerular epithelial cell The continuity between the cistern and the channels of the endoplasmic reticulum is seen (arrow) One of these can be followed to the cell membrane

by a smooth double membrane Contained within this membrane was a loose flocculent material and a varying number of electron dense, homogenous bodies, which were circular on section

Lamellated bodies in the basement membrane like material of mesangial regions were identified as rounded bodies, the interior of which appeared as a more or less complicated pattern, often with a regular arrangement (Fig 11) There was no membrane delineating them towards the basement membrane material Great numbers of somewhat similar smaller bodies appearing with an electron dense periphery, but without internal structure were found in many cases and it was not found worthwhile to quantitate them Therefore, only bodies with some internal structure were counted in the present series

The moon crater like formations appeared on section as pairs of spikes projecting on the epithelial side of the basement membrane (Fig 7 8) These structures, termed major craters, were tabulated when a pair of spikes or one localized mound shaped projection (cut in the periphery of a crater) was seen on the micrograph Another, and not so easily identifiable structure in the peripheral basement membrane was also ennumerated namely minor craters They appeared as small indentations in the epithelial side of the basement membrane, often with a small mass of electron dense material in the basement membrane immediately beneath the crater (Fig 9–10)

In comparing cell counts in the different groups of patients Student T test was applied Frequency of cell organelles and structures in the basement membrane material were compared with a Wilcoxon s rank sum test. In both tests a 5 per cent level of significance was used

RESULTS

Cell Counts

The glomerular cross sections used in the present study varied in size depending on the localization of the section with respect to the glomerular equatorial plane The size of the glomerular cross sections in μ^2 appear in Table 1 The table also shows the range and mean values for the total number of cell nuclei per cross section in each group of patients

The percentage distribution of the 3 cell types in the various groups of patients is seen in Table 2 The differential cell count was identical in all groups of patients

Cell counts were compared in glomerular cross sections grouped after size as shown in Table 3 It was found that differences between small and large cross sections were small There was, however a statistically significant ($2p < 0.01$) increase in the relative number of endothelial cells and a decrease in epithelial cells when large cross sections were compared with the smallest ones The same held true when the individual groups of patients were studied separately

In order to ascertain whether localization of the section with respect to the vascular pole influenced cell counts, these were compared between cross sections containing the vascular pole and those outside the pole region Purposely, cross sections contaning the pole made up the great majority In all of the groups, cross sections with the vascular pole had relatively more mesangial cells and

TABLE 1 *Photomontages of Glomerular Cross Sections*

patient group	no of patients	no of glomeruli	size of Bowman s space μ range	size of Bowman s space μ mean	total number of nuclei per cross section range	total number of nuclei per cross section mean
ND	6	17	7108 24370	16175	68 180	110
D I	7	38	7207–21843	14498	46–143	94
D II	5	17	9431 22866	13550	58 137	95
D III	8	31	7293 28834	15172	37 132	87

Bowman s space was calculated from diameters measured in cm to the inside of the basement membrane of Bowman s capsule ND non-diabetics D I recently diagnosed juvenile diabetics, biopsied both before and after the institution of therapy (3–4 weeks interval) D II patients with 1½ 2½ years duration of diabetes D III patients with 3½ 5 years duration of diabetes

TABLE 2 *Percentage Distribution of 3 Glomerular Cell Types*

uent oup	endothelial cells, % mean	SD of biopsies	SD of glomeruli	mesangial cells % mean	SD of biopsies	SD of glomeruli	epithelial cells, % mean	SD of biopsies	SD of glomeruli
D	38 1	5 5	5 7	30 2	7 8	5 0	31 8	12 1†	6 9
I a*	37 9	4 8	5 0	30 4	5 5	5 7	31 7	5 9	5 6
I b*	38 2	4 4	4 6	29 3	6 6	6 0	32 5	5 8	7 0
II	37 5	4 9	4 7	27 9	5 1	4 5	34 6	8 5	5 9
III	39 0	1 9§	6 1	28 1	4 3	5 9	32 7	4 6	8 2

§ variance less than in other groups
† variance greater than in groups D I b and D III
* biopsy obtained before treatment
* biopsy obtained after treatment
An estimate of the inter individual variation within each group is given with the SD of biopsies, and of the intra individual variation with the SD of glomeruli

TABLE 3 *Percentage Distribution of 3 Cell Types in Cross Sections Ranged after Size*

size of Bowman s space μ^2	no of cross sections	no of biopsies	endothelial cells % ± SD	mesangial cells, % ± SD	epithelial cells, % ± SD
< 13 000	44	22	37 8±5 1	29 7±6 6	32 5±9 2
13 18 000	51	23	38 2±4 1	28 6±4 7	33 1±6 1
> 18 000	23	18	40 3±5 0*	31 2±5 7	28 5±6 6*

* significantly different from small cross sections (2p < 0 01)
In the rangeing of cross sections after size some glomeruli are represented with 2 or more cross sections obtained at different levels of the glomerulus The total number of cross sections therefore is larger than the number of glomeruli studied The number of biopsies appearing in the table is also larger than the total number of biopsies studied, since one biopsy may be represented in more than one size group

TABLE 4 *Percentage Distribution of 3 Cell Types in Cross Sections Containing the Vascular Pole and in Cross Sections Outside the Pole Region*

vascular pole	no of cross sections	no of biopsies	endothelial cells % ± SD	mesangial cells % ± SD	epithelial cells % ± SD
+	87	32	38 4±4 4	29 6±5 1	31 9±6 5
—	31	18	37 4±4 0	25 7±6 3	36 8±7 5*

* significantly different from cross sections with the vascular pole (2p < 0 01)
In some glomeruli cross sections were obtained both at the vascular pole and at a level outside the pole region This accounts for the large number of cross sections

TABLE 5 *Number of Cells per 1000 µ² of Bowman s Space*

	endothelial cells ± SD	mesangial cells ± SD	epithelial cells ± SD	total cell count ± SD
ND	2 7±0 6	2 2±0 8	2 2±0 9	7 1±1 3
D I	2 5±0 5	1 9±0 5	2 1±0 6	6 6±1 2
D II	2 6±0 4	2 0±0 6	2 5±1 0	7 1±1 5
D III	2 3±0 3	1 7±0 2	2 0±0 5	6 0±0 7

In group D I results are included from 2 biops es obtained before and after treatment

TABLE 6 *Number of Mesangial Cells pr 100 µ² Mesangial Area*

	No of glomerular cross sections	No of mesangial cells per 100 µ² mesangial area ± SD
ND	12	1 4±0 2
D I	16	1 2±0 7
D II	12	1 3±0 2
D III	16	1 1±0 5

The table comprises 56 cross sections in which the total area of mesangial regions was measured with planimetry

fewer epithelial cells compared with the remaining cross sections Overall results from all of the groups appear in Table 4 Only the percentage contributed by epithelial cells differed significantly ($2p < 0\,01$) in the two subgroups of glomerular cross sections

The results of calculations of the density of cells appear from Table 5 Hyperplasia was not observed in any cell type in the diabetic patients In a total of 56 glomerular cross sections, all of which contained the vascular pole, the size of mesangial regions was measured by planimetry (42) In these cases the



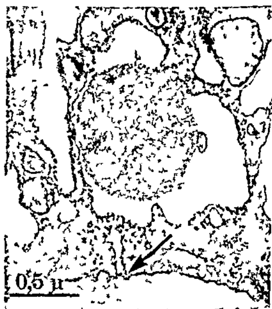


Fig 2 + 3 The micrographs show an ERC in serial sections (no 1 and no 9 in a ribbon of sections) In Fig 2 the connection between the cistern and the endoplasmic reticulum is seen at the arrow This part of the reticulum was followed via intervening sections (not shown) to a location in a foot process close to the basement membrane at the arrow in Fig 3

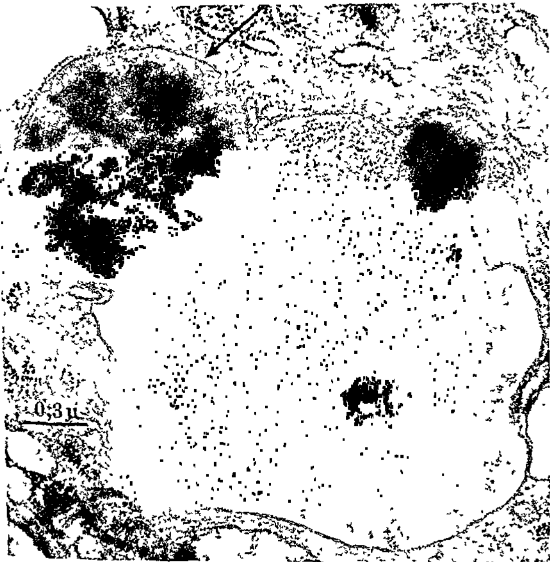


Fig 4 Heterogeneous inclusion body of an epithelial cell It contains a flocculent material and dense bodies, two of which are protruding The limiting double membrane can be followed to the surface of the large, dense body (arrow)

number of mesangial cells per unit mesangial area was calculated As shown in Table 6 there was clearly no hyperplasia of mesangial cells in the groups of diabetics

Cell Organelles

Cisternae in the endoplasmic reticulum (ERC) containing basement membrane-like material were observed occasionally with an average frequency of about one such organelle per cross section The diameter of this organelle was usually 5–10 000 Å The basement membrane-like material in the interior of the cisterna practically always appeared as rounded, circular or elliptical bodies on section In some cases the direct continuity between the ERC and the flat channels of the endoplasmic reticulum was seen (Fig 1) In many cases this organelle was photographed in a number of serial sections in



Fig 5 A cistern in the endoplasmic reticulum the membrane of which is in close contact with the membrane of the heterogeneous inclusion body at the arrow

order to follow the communication via the channels of the endoplasmic reticulum Often the connecting channels could be traced to the perinuclear space or the vicinity of the cell membrane Fusion of the membranes, however, could not be demonstrated On only one occasion was communication established to a location very close to the basement membrane (Fig 2 and 3) Since the number of serial sections obtainable in each case was usually small no certain conclusions can be reached on this point

The heterogeneous inclusion bodies (HB) in epithelial cells occurred more frequently The dense bodies of varying size contained within the inclusion bodies were often seen protruding on the surface In these cases the limiting double membrane could be followed around the outer circumference of the protruding body (Fig 4) Quite often mitochondria were seen in close proximity to the HB However, this association was not quantitated, and it is uncertain whether the finding was more frequent than could be expected by chance It appears from Table 7, that the average number of these organelles (ERC and HB) per 10 000 μ^2 was higher in the groups of diabetics with some years' duration of disease However, the difference between the average values in diabetics in groups II and III on the one hand, and that of the control group on the other, was not statistically different It was due to very large numbers in some of the diabetic patients

Figures 5 and 6 show a frequent finding, the two types of organelles occurring together either in direct contact as illustrated or very close together This was the case in about one fourth of the total number of ERC observed *(32 out of 129)*

TABLE 7 *Frequency of Cell Organelles (ERC and HB) and Substructures in the Basement Membrane*

	ERC		HB		Major Craters		Minor Craters		Laminated Bodies	
	no per glom	no per 10 000 μ^2	no per glom	no per 10 000 μ^2	no per glom	no per 10 000 μ^2	no per glom	no per 10 000 μ^2	no per glom.	no per 10 000 μ^2
	0 8	0 5	10 0	6 0	1 3	0 8	5 5	3 4	0 4	0 3
	0 9	0 7	7 3	4 9	1 3	0 9	6 3	4 3	0 4	0 3
I	0 9	0 7	9 6	7 2	2 5	1 9	8 5	6 2	1 4*	0 9*
II	1 6	1 1	12 1	7 9	2 6	1 6	10 0	5 9	2 3*	1 4*

*S gnif cantly different from controls (2 p < 0 01)
Cisternae in the endoplasmic reticulum (ERC), heterogeneous inclusion bodies (HB), craters on the basement membrane and laminated bodies in the basement membrane like material were counted on each of the cross sections studied and the prevalence per unit of Bowman s space was calculated

Structures in the Basement Membrane

The morphology of the major craters was similar in diabetic and non diabetic patients The spikes were usually blunted In the substance in between two spikes whorled, banded fibers with a cross diameter of about 550 Å were often seen showing a cross striation with a periodicity of 140 Å (Fig 7) The basement membrane substance located between these bands and the epithelial cell frequently showed rarefaction (Fig 8) In the area between *the two spikes filtration slits were always ab sent* The cytoplasm of the epithelial cell immediately adjacent to the crater often had densely packed cytoplasmic fibrils



Fig 6 At the site of contact between the two organelles (ERC and HB) the limiting membranes can not be dissolved probably because they are not perpendicular to the plane of section

The average number of major craters for each biopsy was between 1–3 It is seen from Table 7, that the frequency is identical in *controls and recently diagnosed diabetics*

The minor craters although quite well-defined were rather inconspicious (Fig 9–10) They always occurred with a greater fre quency than the major craters Results of the counts are shown in Table 7 They were ob served more frequently in diabetics in groups II and III compared with non diabetics and recently diagnosed diabetics The differences between the groups however, *were not signif icant*

A characteristic *laminated body* is shown in Fig 11 These structures were located in the basement membrane like material in mesangial regions and were not observed in the peripheral basement membrane At times *knife marks* appeared to originate at such *bodies* Table 7 shows that they were observed with increased frequency in diabetics in



Fig 7 Sect on through a moon-crater like formation on the epithel al s de of the basement membrane In the basement membrane substance a whorled banded fiber with 140 Å period city is seen

groups II and III The difference between these groups and non diabetics was significant (2 p < 0 05)

DISCUSSION

Counts of cells and cellular structures at the ultrastructural level in glomeruli have not previously been reported This high resolution permits a precise definition of each cell type and provides precise figures for the d stribu tion of cells and of cell density The technique is very time consuming thus limiting the number of cross sections that can be quan titated

The number of cells found in the present study per unit area of Bowman s space (7 cell nuclei per 1000 µ) is in good agreement with light mocroscop c findings in control subjects reported by *Iidaka et al* (1968) (about 6/1000 µ) and *Hanberg Sorensen* (1972) (7 8/1000 µ) *Wehner & Anders* (1970) on the other hand reported total cell counts of about 30 cells per cross sections of about 19 000 µ Their figures therefore are far from agreement with the remaining reports

The percentage distribution of the three cell types found in the present electron microscopic study shows sl ght differences from those observed at light microscopy Re sults from Kimmelstiel s group (18 21) as well as those of *Hanberg Sorensen* (1972) showed a higher incidence of endothel al cells (43–44 per cent) Apparently distinct on between endothelial and mesangial cells both of which are located inside the PAS positive basement membrane material cannot always be made with light microscopy *Hanberg So rensen* (1972) preferentially selected sections which did not include the vascular pole This may partly explain his lower percentage of mesangial cells There still remains however,

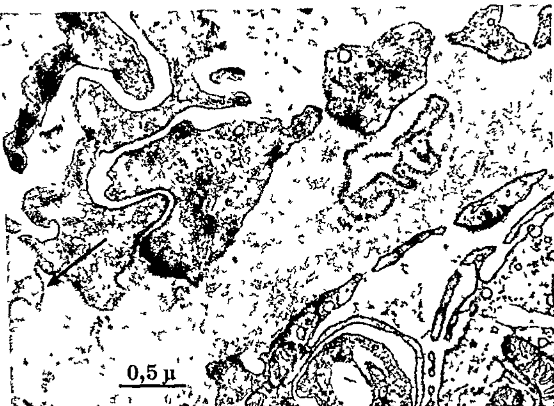


Fig 8 Moon crater formation The rarefaction in the basement membrane substance between the whorled fiber and the epithelial border of the basement membrane is evident In the basement membrane-like material of the mesangial region a similar banded fiber is seen at the arrow

discrepancies in the percentages of endothelial and epithelial cells in these two studies The differences are not large and presumably are due to the very different degrees of resolution in the two studies *Wehner & Anders* (1970), on the other hand in their differential cell counts found a much lower incidence of mesangial cells (16 per cent) and a much higher incidence of epithelial cells (48 per cent) This variance from the present results is unexplainable

In light microscopic studies (17 18) it was recommended that the most peripheral sections of glomeruli not be used for cell counts because they give more variable results than the remainder of the glomerulus In the present study the smallest glomerular cross sections used had a diameter about half that of the largest This means roughly, that the central 80 per cent of a cross section is used According to Hanberg s examples this is within the space of least variation

The location of the glomeruli used in the present study with respect to kidney cortex or medulla is undeterminable Results of the light microscopic study reported by *Hanberg Sorensen* (1972) however, showed no differences in cell counts or cell density between different locations in the kidney

The present results of cell counts in patients with short term diabetes clearly showed no differences between diabetic and control patients in percentage distribution or density of cells This contradicts the observation that mesangial cell hyperplasia is a very early event (7 13, 14) Since thickening of the peripheral basement membrane and increase in mesangial basement membrane like material was present in the diabetic patients with 1½–5 years duration the idea is untenable

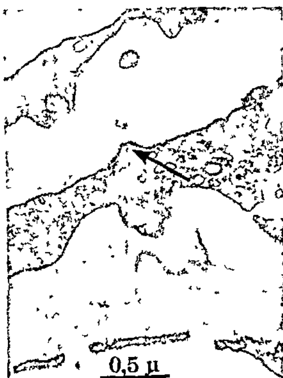


Fig 9 A minor crater in the basement membrane (arrow), located immediately opposite a major crater in another loop

that mesangial cell hyperplasia is the cause of increased production of basement membrane as has been suggested by some authors (7, 13 14, 22) The thesis of *Wehner* & *Anders* (1970) based on their finding of mesangial cell hyperplasia concerning an inflammatory genesis for the diabetic changes, is also left without support Results at variance with those of the above mentioned studies were also reported by *Fukuhara* (1968) in his study of diabetic glomeruli with slight diffuse enlargement of mesangial regions He found normal or decreased density of mesangial cells within the mesangial regions

For tentative speculations on the pathogenesis of diabetic glomerular lesions however morphological aspects of the functional state of the glomerular cells seem to be of much greater interest than their absolute number The degree of cellular activity is presumably reflected in the appearance of various cell organelles The state of these organelle systems in early diabetes is therefore of great interest Deviations from normal, in particular increased amounts of organelles have been reported in patients with diabetic glomerulopathy (6, 7, 19, 22) It is, however, very difficult to quantitate the organelles The large variations from one cell to another and from one location to another within the cell cytoplasm, obviously make non quantitative statements rather senseless

The two types of cell organelles counted in the present study might be of some interest with respect to diabetic glomerulopathy Since the organelle is part of the endoplasmic reticulum, it contains material synthesized by the epithelial cell In an experimental study (1) it was shown that the content of the cisterna and the basement membrane share common antigens Thus strong evidence is at hand that the organelle is involved in basement membrane synthesis Studies with a silver-labelling technique (23, 24, 40) provide further evidence that the epithelial cells —and probably also the mesangial cells—, participate in basement membrane synthesis

The cisternae in the endoplasmic reticulum seem to have attracted little attention in human biopsies This is not surprising when



Fig 10 Two minor craters on the epithelial side of a basement membrane Beneath the slight indentations in the epithelial aspect of the basement membrane surface condensations are seen in the basement membrane structure

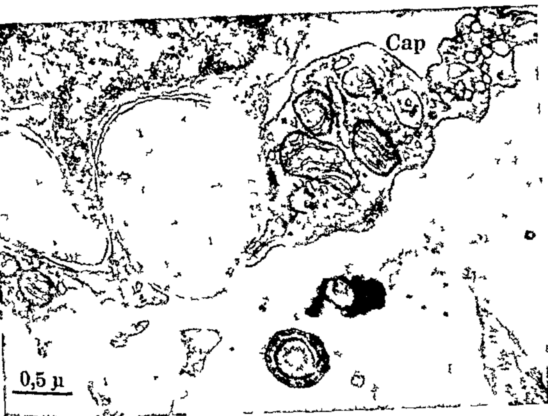


Fig 11 Pa of a me ang al eg on s n he lowermost part of tl e p cture In the basement memb ane l ke ma erial one typ cal lam na ed body s seen Cap cap lary lumen

the r pre alence of 1 per cross sect on is taken n o account. In the present study the con tent of tl e c sternae was al vays found to be f nely f lamentous The same f ne structure was illustrated by *Trun p et al* (1962) in one human case and by *Tl oenes* (1967) n an other human b opsy The remain ng reports on this t pe of organelle are all from stud es in rats (1 11 1? 37) In th s spec es the or ganelle occurs more frequently (11 12 31) and ts content is found to be nhomogeneous l gh er and darker areas alternat ng the latter some mes sho v a l near pattern Period city as not observed in tl e present study Com mun cat on from the c sterna to the cell sur face and n one case not far from the base ment membrane vas documented in the pre sent ser es Ho ve er tl e mechan sm by h ch the synthes zed material s extruded from tl e cell is not known

Al hough no s gn f cant d fferences in the prevalence of endoplasm c ret culum c sternae were observed n any of the groups the ob v ous tendency towards greater numbers n the d abetics n groups II and III does de serve some interest. Larger series of pat ents will have to be stud ed to determ ne whether the tendency will turn out to become s gn f cant However even in the case of pos t ve f ndings the nterpretat on must be caut ous A greater number of c sternae may not be equ valent w th an increased rate of base ment membrane synthes s. The organelles m ght on the contrary increase n number f release of basement membrane substance pro duced in the endoplasm c ret culum is im peded

The he erogeneous inclus on bodies be cause of their greater prevalence are seen more often in llustrations and they have been ment oned by some authors as occurr ng n both control subjects (4 20) and patho

logic states (5, 6, 9, 33) Their functional significance is not known They have been termed lipid droplets hyaline droplets opaque bodies One possibility is that these bodies represent material reabsorbed by the epithelial cells as suggested by *Jorgensen* (1966) Since similar structures are apparantly not observed in laboratory animals, the experimental studies of glomerular filtration do not aid in the solution of the problem If this hypothesis is correct then the HB would be expected to be found with increased frequency in cases of increased glomerular permeability No data on this problem are available The patients in the present series with short term diabetes did not have proteinuria It has been shown in clearance studies (26) that diabetic patients of this category do not evidence altered glomerular permeability Theoretically however, increased permeability of the glomerular filter may have been present, which was completely compensated by increased epithelial reabsorption This in turn could be reflected in the very high number of HB observed in some of the patients in group III

The close association between ERC and HB observed in many cases could suggest that some of the reabsorbed material may be used by the cell as raw material for the synthesis of basement membrane substance This purely hypothetical pathway needs to be investigated in functional studies

The significance of the moon crater like formations also remains speculative In a preliminary report we suggested that the craters represented sites where newly synthesized basement membrane material was being layed down The present material does not provide further evidence for this theory The present extended study of the craters in human biopsies confirmed that craters are present with increased frequency in some of the diabetic patients in group III although the frequency was not significantly increased in the group as a whole A new observation is the whorled banded fibrils with 140 A periodicity seen in many cases in connection with the crater formation Similar fibrils are observed in the basement membrane like material in mesangial regions in patients with diabetic glomerulopathy They have been illustrated by other authors (9, 10) and have been interpreted as immature collagen Similar structures have been described in Bruch s membrane in one patient with diabetic retinopathy (25) and were interpreted as a sign of basement membrane degeneration The banded fibers may represent an unusual degree of polymerization of the basement membrane glycoproteins perhaps at sites of newly layed down material The dense material sometimes observed in the minor craters did not show periodicity (The count of these structures was due to their inconspiciousness somewhat more uncertain than that of the other structures)

Laminated bodies similar to those observed in the present study have previously been described in cases of hepatic glomerulosclerosis (28) and in a patient with argyria (27) in which case they were interpreted as silver containing bodies In the present study they were observed occasionally in the control group so that the difference between the controls and diabetics is a quantitative one In some cases the content of these bodies appears to be hard since knife marks are produced

The ultrastructure of diabetic glomerulopathy is principally characterized by increased amounts of basement membrane both in mesangial regions and in the walls of the glomerular capillaries The present quantitative data on some of the glomerular structures do not give any definitive answers with regard to the pathogenesis of basement membrane abnormalities The functional counterpart of many of the observations cannot be evaluated However the study showed that cellular hyperplasia does not play a causative role On the basis of the finding of a tendency to increased numbers of ERC and carters it might tentatively be suggested that increased basement membrane synthesis is the cellular reaction to some abnormal condition (e g high blood glucose insulin deficiency high growth hormone) The studies of basement

membrane biochemestry in diabetes mellitus by Spiro and coworkers (3, 30) have opened a new field for the further investigation of diabetic angiopathy. In the time to come a coordination of biochemical and morphological studies on basement membranes, and also on subcellular structures such as those dealt with in the present paper, may prove to be fruitful as regards elucidation of the pathogenesis of diabetic angiopathy.

My thanks are due to Drs *G. Gregersen*, *H. Skjoldborg*, and *B. Møller* for the kidney biopsies, and to Dr *H. J. Gundersen* for statistical aid.

The skilful technical assistance of Mrs *Kirsten Gerlach*, *Helle Danvad Jensen* and *Helen Hou Jørgensen* is gratefully acknowledged.

The present study was supported by grants from *Statens lægevidenskabelige Forskningsråd*, *Michaelsen fonden*, *Novo fonden*, *og Rask Ørsted fonden*.

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CORRESPONDENCE BETWEEN GLOMERULAR SIZE AND RENAL VOLUMES IN ATROPHIC, NORMAL, AND HYPERTROPHIC HUMAN KIDNEYS

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The following components of total renal volume were quantitated in 14 human kidneys affected by generalized atrophic or hypertrophic conditions superficial cortex, columns of Bertin, medulla and pelvis These components were found to be present in proportions that were quite constant and essentially the same as those previously found in normal kidneys *Thus all parts of the kidney are equally responsive to generalized atrophic or hypertrophic conditions* In normal as well as abnormal kidneys the mean glomerular diameter was found to be related to total renal (or cortical) volume, but in highly different manners for normal and abnormal kidneys Glomeruli of greatest diameter were found in kidneys having high demands for renal function in hypertrophic abnormal kidneys, or in normal kidneys of small renal volume, having increased functional demand per nephron Smallest glomeruli were observed in kidneys with low functional demand in atrophic abnormal kidneys or in normal kidneys of greatest renal volume, having small functional demand per nephron.

In a recent study of normal human kidneys, it was found that the cortical, medullary, and pelvic fractions of the renal parenchyma are present in characteristic and relatively constant proportions, independent of the renal volume (*Hegedus & Faarup* 1972) Evaluation of similar parameters in abnormal kid neys has not yet been undertaken

In addition, no report of changes in glom erular size reflecting quantitative changes in renal volume is available at present

Received 19 v 72

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The purpose of the present paper is to determine whether systematic, parallel changes in glomerular size and renal volume are found in normal as well as abnormal human kidneys

MATERIALS AND METHODS

Materials

*Normal kidneys** Twelve human kidneys were obtained from 7 autopsies, 6 kidneys were from males 6 from females Patient age ranged from 24

* The normal kidneys used in this study for correlation with glomerular size were also used for fractional volume determination by *Hegedus & Faarup* (1972)

TABLE 1 *Abnormal Kidneys Used in Volume Estimations*

Kidney number	Age	Sex	Left/right	Hypertension	Total renal volume (cm^3)	Pathology
H	68	♀	R	+	50	Hydronephrosis Valve at uretero pelvic junction
1	–	–	L		240	Compensatory hypertrophy
2	67	♂	R	+	70	Old thrombus in renal artery
3	–	–	L		235	Compensatory hypertrophy
4	68	♂	L	?	85	Atherosclerotic stenosis of renal artery
5	–	–	R	–	205	Compensatory hypertrophy
6	50	♂	L	0	95	Renal artery and ureter steno by pulmonary CA metastases Mild hydronephrosis
7	–	–	R	–	205	Compensatory hypertrophy
H	81	♂	R	+	55	Hydronephrosis
8	–	–	L	–	185	Compensatory hypertrophy
9	73	♀	R	0	75	Atherosclerotic stenosis of renal artery
10	–	–	L	–	140	Compensatory hypertrophy
11*	29	♀	L	+	60	Renal artery stenosis
H	73	♀	L	+	55	Hydronephrosis resulting from calculi
12	–	–	R	–	240	Compensatory hypertrophy
13	66	♀	L	+	70	Atherosclerotic stenosis of renal artery
14	–	–	R	–	180	Compensatory hypertrophy

H = Kidneys with severe hydronephrosis (considered to be non functional), prohibiting fractional volume and glomerular investigations

While the contralateral, hypertrophic kidneys were used in the study, the hydronephrotic ones were not used, but were included in the table for informational purposes only

* Surgical specimen (one sided nephrectomy)

to 70 years In no patient was there historical clinical or laboratory evidence of kidney disease or of hypertension and the absence of pathological changes was confirmed microscopically Each kidney was supplied by a single renal artery and vein and drained by a single ureter

Abnormal kidneys Fourteen human kidneys were obtained from 8 autopsies 1 surgical specimen (nephrectomy) is included in this group Nine kidneys came from males 5 from females Patient age ranged between 29 and 81 years Each kidney possessed as in the normal group only a single renal artery vein and ureter The pathological changes found in the group of abnormal kidneys are noted in Table 1

Methods

Fractional volume determination The normal as well as the abnormal kidneys were fixed in 10 per cent formalin solution for a minimum period of 4 days after which weight and total renal volume (by Archimedes principle) were determined Each kidney was sectioned perpendicular to its long axis into parallel slices 1 cm in thickness the cut surface of each slice was photographed to permit planimetric measurement of the fractional renal volumes By this method the following components of total renal volume were quantified (Fig 1) 1 Total cortical volume, subdivided into a superficial cortex' (not including the columns of Bertin) and b columns of Bertin, 2 medullary volume, and 3 sinus and pelvic volume (renal pelvis plus the fat tissue in the sinus) Fig 2 illustrates the appearance of the sectioned kidneys prior to planimetry

Glomerular diameter determination From each normal and each abnormal kidney, 3 sections of renal cortex were taken for microscopic examination and were PAS stained Two of these sections were from the superficial cortex, section "A" from

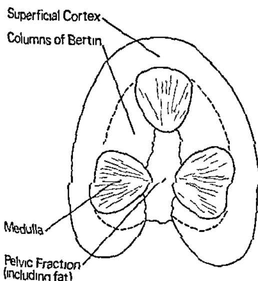


Fig 1 A schematic representation of the cut kidney surface showing the composition of the fractional renal volumes measured by planimetry

superficial cortex opposite the hilus and section C from superficial cortex at one of the poles The third section section B consisted of cortical tissue exclusively from the columns of Bertin Each of these 3 microscopic sections was further subdivided into 3 regions sections A and C into external middle and deep regions of superficial cortex section B into 2 juxtamedullary and one central regions of the columns of Bertin (Fig 3) In each of the three regions of each section the diameters of 25 glomeruli were measured using an eyepiece micrometer A total of 225 glomerular diameters was measured for each kidney The diameters of glomeruli which appeared excessively small were not measured as such glomeruli were resultants of highly tangential sectioning

Glomerular concentration determination For the normal kidneys the 3 cortical sections A B and C (Fig 3) were subdivided into 3 regions as above Each of the 3 regions of each section was examined under 100× magnification and the number of glomeruli per microscopic field was counted All glomeruli that were not entirely visible within a field were counted as one half a glomerulus no

Fig 2 A kidney cut into parallel slices 1 cm in thickness showing each component of total renal volume superficial cortex columns of Bertin medulla and sinus and pelvis

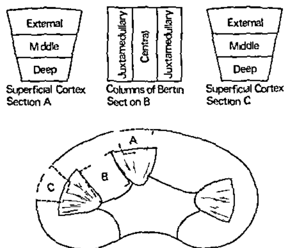


Fig 3 Origins of the cortical sections taken for microscopy Section A from superficial cortex opposite the hilus section B from the columns of Bertin and section C from superficial cortex at one of the renal poles Each of these sections is shown above subdivided into 3 cortical regions

attempt was made to estimate fractions of glomeruli seen Nine different fields were viewed in each cortical subdivision thus glomeruli were counted in 81 fields in each of the normal kidneys The diameter of the field was 1150 microns

RESULTS

Normal Kidneys

The correlation between glomerular diameter and renal volume The mean value of 75 glomerular diameters measured in each of the cortical sections A, B and C (Fig 3) is compared with the total renal volume for the group of normal kidneys in Fig 4 Two conclusions are appearent from this figure First the mean glomerular diameter is related to total renal volume, though in a non linear fashion Normal kidneys having smallest renal volumes contain glomeruli with the greatest diameters those of greatest renal volume exhibit smallest glomeruli Secondly, no constant relationship was found between glomerular diameter and the portion of cortical tissue examined, thus the glomerular diameter does not vary systematically between superficial cortex opposite the hilus at one renal pole or in the columns of Bertin when using the technique presented above (Table 3) In addition there was found to be no statistically systematic variation between the mean glomerular diameter and the region of cortex examined ($p > 0.05$), thus within a single normal kidney, the mean glomerular diameter is statistically equivalent in external middle, and deep regions of the superficial cortex and in juxtamedullary and central regions of the columns of Bertin The relationship between mean glomerular diameter and total cortical volume is much the same as that for total renal volume due to the constant linear relationship between total cortical volume and renal volume

Lack of correlation between renal volume and glomerular concentration In normal kid

TABLE 2 *Minimum Maximum and Mean Contributions to the Total Renal Volume Expressed as Percentage Volumes by Each Renal Fraction for Normal and Abnormal Kidneys*

	Normal kidneys			Abnormal kidneys		
	Min %	Max %	Mean %	Min %	Max %	Mean %
Total cortical volume	51 7	63 3	56 1	55 5	66 0	60 0
Superficial cortical vol	38 7	51 2	43 4	30 9	49 0	38 6
Columns of Bertin volume	5 9	17 0	12 7	16 5	28 2	21 9
Medullary volume	26 1	38 5	32 0	19 3	33 3	26 7
Sinus and pelvic volume	8 5	19 1	11 9	7 0	21 3	13 2

Values for normal kidneys are from *Hegedus & Faarup* (1972) The total cortical medullary and sinus and pelvic volumes were found to be essentially identical in per cent in the abnormal (atrophic as well as hypertrophic) and the normal kidneys

TABLE 3 *Mean Glomerular Diameter Related to Position in Cortex (in Microns)*

		Superficial cortex			Columns of Bertin	
		External	Middle	Deep	Central	Juxta-medullary
Normal kidneys	1	175 3	166 9	164 7	176 4	171 1
	2	171 3	166 7	163 8	158 6	156 1
	3	224 6	230 4	204 1	186 1	190 5
	4	181 4	170 9	169 1	171 1	172 4
	5	212 1	206 1	201 6	203 9	213 0
	6	151 2	150 0	154 4	140 8	145 3
	7	173 3	180 8	177 0	187 7	182 7
	8	158 7	159 4	168 8	174 9	175 0
	9	168 2	170 9	170 2	162 3	166 4
	10	168 0	171 3	176 9	157 3	160 5
	11	173 4	169 5	169 9	188 9	181 7
	12	152 3	146 9	149 0	140 4	145 0
Abnormal kidneys	1	192 4	181 0	176 3	182 7	178 5
	2	174 1	173 0	174 1	168 9	172 5
	3	214 1	216 5	212 7	220 5	209 9
	4	137 0	153 4	152 2	147 0	152 3
	5	166 7	179 5	184 7	178 9	172 4
	6	168 6	152 5	155 9	158 9	161 4
	7	219 6	218 2	207 2	214 0	222 4
	8	191 3	176 7	169 5	164 8	180 5
	9	173 8	172 7	159 5	–	171 9
	10	159 5	156 7	161 7	–	188 7
	11	146 7	139 4	145 1	132 0	140 0
	12	182 7	167 8	164 5	190 2	188 1
	13	150 0	165 5	171 1		159 8
	14	196 3	183 9	181 4	198 0	193 5

Values for external, middle, and deep regions of the cortex were obtained by taking the mean glomerular diameter in sections A and C (*cf* Fig 3) Columns of Bertin values represent the mean value of the two juxtamedullary regions of section B and a separate value (termed *central*) for the central region

neys, the mean number of glomeruli per microscopic field in each cortical section A, B, and C was compared with the total renal volume (Fig 5) As can be seen from the figure, and as substantiated statistically (r_s = 0 3934), there is no significant correlation between the number of glomeruli per field and the total renal volume by the technique applied.

Abnormal Kidneys

Fractional volume determination In the group of atrophic and hypertrophic kidneys, the percentage contributions to total renal volume made by cortical, medullary, and sinus and pelvic fractions were determined by planimetry In Fig 6 these findings are summarized and it is seen that the abnormal kidneys do not differ substantially from the normal group in mean values for 1 total cortical percentage, 2 superficial cortical percentage, 3 columns of Bertin percentage, 4 medullary percentage, or 5 sinus and pelvic percentage (also see Table 2) In addition, no substantial difference in fractional volume percentages was observed when atrophic and hypertrophic kidneys were compared Furthermore, when the cortical volume was measured separately for the superficial cortex and for the columns of Bertin (Fig 7), the two component cortical volumes were found to be inversely related Thus kid-

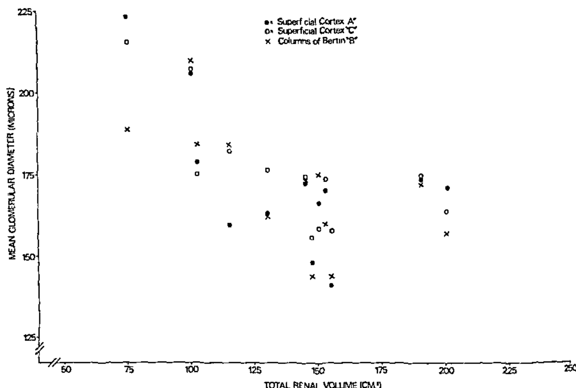


Fig 4 The mean glomerular diameter in section A (●) of superficial cortex opposite the hilus, in section B (×) from the columns of Bertin and in section C (○) of superficial cortex from one renal pole is compared with total renal volume (normal kidneys only) A non linear, inverse relationship is present for the normal kidneys There is no correlation between glomerular diameter and the area of cortex examined

neys having large superficial cortical volumes exhibit small columns of Bertin, and *vice versa*

Correlation between renal volume and glomerular diameter The mean value of the 75 glomerular diameters measured in each of the cortical sections A, B and C (see Fig 3) was compared with the total renal volume for the group of abnormal kidneys (Fig 8) The absence of the inverse relationship between glomerular diameter and renal volume, as was found in the normal kidneys, is readily appearent Instead, abnormal kidneys with the smallest renal volumes (atrophic kidneys) exhibit glomeruli with the smallest diameters those with the largest renal volumes (hypertrophic kidneys) have the largest glomeruli Thus the relationship between renal volume and glomerular diameter is clearly different in normal and in abnormal kidneys, as is seen directly from Fig 9 in which the average glomerular diameter is compared with the total renal volume for both normal and abnormal kidneys

In other respects, the findings in abnormal and normal kidneys were similar First, glomerular diameter showed no systematic variation between cortical sections A, B, and C In addition, no systematic variation was found in glomerular diameter within the subdivisions of sections A, B, or C (in external, middle, and deep regions of superficial cortex, or in central and juxtamedullary regions of the columns of Bertin) (Table 3) Finally, the relationship between glomerular diameter and total cortical volume was found to be much the same as that obtained using total renal volume (Fig 9) due to the constant linear relationship between the two parameters

●= Superficial Cortex "A"
○= Superficial Cortex "C"
×= Columns of Bertin "B"

MEAN NUMBER OF GLOMERULI PER FIELD

TOTAL RENAL VOLUME (CM³)

Fig 5 The mean number of glomeruli per field is contrasted with total renal volume for each section of cortex in section A (●) of superficial cortex opposite the hilus, in section B (×) from the columns of Bertin, and in section C (○) of superficial cortex from one renal pole for the group of normal kidneys No correlation was found between these two parameters.

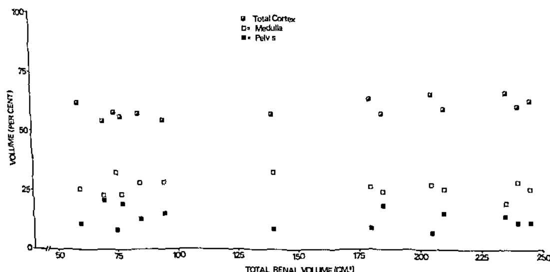


Fig 6 The percentage contributions to total renal volume by the total cortical (◪), medullary (□), and sinus and pelvic (■) volumes in abnormal kidneys Both atrophic and hypertrophic kidneys were found to possess fractional volumes in proportions essentially identical to those found in normal kidneys

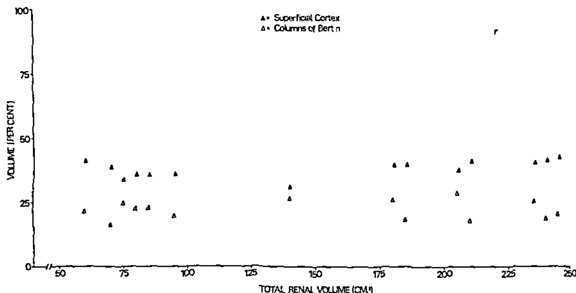


Fig 7 The percentage contributions to total renal volume of the superficial cortical (▲) and columns of Bertin (△) volumes in abnormal kidneys Both atrophic and hypertrophic kidneys show an inverse relationship between these two component cortical volumes, kidneys having large percentage volumes for the superficial cortex have small columns of Bertin, and *vice versa*

●= Superficial Cortex "A"
o= Superficial Cortex "C"
×= Columns of Bertin "B"

MEAN GLOMERULAR DIAMETER (MICRONS)

225
200
175
150
125

50 75 100 125 150 175 200 225 250

TOTAL RENAL VOLUME (CM³)

Fig 8 The mean glomerular diameter in section A (●) of superficial cortex opposite the hilus in section B (×) from the columns of Bertin, and in section C (○) of superficial cortex from one renal pole in compared with total renal volume Abnormal kidneys only The relationship found is clearly different from that present in normal kidneys, in which an inverse relationship is present There is no correlation between glomerular diameter and the area of cortex examined

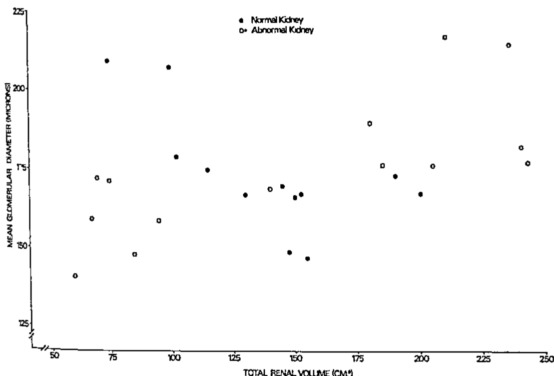


Fig 9 The mean glomerular diameter in each normal (●) and each abnormal (○) kidney is contrasted with total renal volume, showing the different relationships found for the two groups of kidneys One abnormal kidney (volume = 140 cm³) is located among the normals This kidney was only minimally hypertrophic, while the opposite kidney from the same patient was atrophic (volume = 75 cm³)

Glomerular concentration in abnormal kidneys As no correlation was found between the number of glomeruli per field and renal volume in the group of normal kidneys, a similar investigation of such a relationship was not made for the group of abnormal kidneys

DISCUSSION

Normal kidneys The recent study by *Hegedus* & *Faarup* (1972), utilizing both renal angiography in patients and planimetric measurement of autopsy kidneys for estimation of fractional renal volumes, has determined that the components of total renal volume are present in well-defined, relatively constant proportions Aside from a slight increase in the sinus and pelvic fraction with age, due to a proportional increase in fat tissue in the sinus, the cortical, medullary as well as sinus and pelvic fractional renal volumes were shown to be essentially independent of age, side (left or right), and sex in normal kidneys The present study was undertaken to determine whether renal volume, and thus fractional renal volumes, were related to the size of the nephrons, as estimated by glomerular diameter, or to "glomerular concentration", the number of glomeruli per microscopic field

The relationship found between glomerular diameter and renal (or cortical) volume is not a simple linear one (Fig 4) It must be assumed that many factors are responsible for the genesis of a kidney having a given renal volume and containing glomeruli of a certain size Among these factors two appear to be of particular importance First if normal kidneys of identical renal volumes

possess a similar number of nephrons, the number of nephrons should be smallest in kidneys of smallest renal volume, and greatest in kidneys of largest renal volume Secondly, having a fixed number of nephrons in a given renal volume, the amount of renal function necessary for homeostasis is variable from individual to individual, kidneys of equal volumes and equal nephron number may be confronted by highly different functional demands, and must respond with either atrophy or hypertrophy at the histological level Thus, the processes of work hypertrophy and disuse atrophy may be considered physiological responses in normal kidneys, and pathological ones in abnormal kidneys, the difference being quantitative and *not* qualitative

Since the number of nephrons cannot be increased after foetal development, normal kidneys of small renal volumes will be, in general, subject to greater influence by a hypertrophic process resulting from increased functional demands, as shown in Fig 4, these kidneys possess the greatest glomerular diameters At the other extreme, large normal kidneys with greatest renal volumes and probably the highest number of nephrons will be primarily influenced by disuse atrophy, and exhibit the smallest glomeruli This is also seen in Fig 4

The finding of an absence of correlation between renal volume and glomerular concentration is apparently due to inadequacies in technique

Abnormal kidneys The 14 kidneys comprising the abnormal group were selected because, in each case the pathological process responsible for renal atrophy or hypertrophy was one affecting the entire renal parenchyma in a generalized fashion Thus cases of chronic glomerulonephritis, chronic pyelonephritis, or other renal diseases were not included in this study because of the somewhat more uneven distribution of such lesions

The fractional renal volumes obtained for both atrophic and hypertrophic kidneys (Fig 6) are essentially the same as those found in normal kidneys Thus cortical medullary, as well as sinus and pelvic components are all involved in atrophic or hypertrophic processes, and each maintains its characteristic percentage contribution to total renal volume in the pathological state This finding is contrary to very early animal experiments (*Lorenz* 1886, *Nothnagel* 1886), from which it was concluded that hypertrophy primarily increases the size of the cortex, and that the medulla is affected to a lesser degree These experiments were performed on unilaterally nephrectomized dogs, the normal (extirpated) kidney serving as the control with which the hypertrophic kidney was later compared No quantitative measurement of fractional renal volumes was performed, and atrophic kidneys were not investigated

Further, the complementary relationship between the volumes of the superficial cortex and the columns of Bertin (Fig 7) found for the group of abnormal kidneys indicates the need for caution in applying the often used gross description "the renal cortex appears thinned" unless the volume of the columns of Bertin can also be estimated Just as normal kidneys with small superficial cortical volumes will contain more numerous and larger columns of Bertin (*Hegedüs* & *Faarup* 1972), 'abnormal" kidneys with a "thin" superficial cortex may have large columns of Bertin, and thus a normal cortical volume A recent angiographic study of "apparently normal kidneys' having minimally decreased function (*Hegedüs* & *Ravnskov* 1972) collaborates these results, though as noted by the authors rontgenological methods cannot sufficiently quantitate the volume of the columns of Bertin to allow firm conclusions

The correlation between renal volume and glomerular diameter found in both atrophic and hypertrophic kidneys (Fig 8) is clearly different from that found in the normal group Similar glomerular measurements were done with hypertrophic kidneys in unilaterally nephrectomized dogs by *Ribbert* (1884) His conclusions are based on measurements of only 30 glomeruli per kidney, and no attempt was made to correlate the changes in glomerular size with actual renal volumes,

kidneys were placed in either "normal" or 'hypertrophic" groups and glomerular diameter was compared only between the two groups More recently, changes in the diameter, length, and volume of the proximal and distal tubules of hypertrophic kidneys in unilaterally nephrectomized rats have been reported by *Hayslett et al* (1968) using a freeze-drying technique, but this study also lacks information on the magnitude of these changes as related to the degree of hypertrophy The changes in tubular dimensions were compared only between normal and hypertrophy groups, and not directly with actual renal volume

In conclusion, the measurement of component renal volumes has demonstrated that all portions of the renal parenchyma participate nearly equally in atrophic and hypertrophic processes Also, the variance in glomerular diameter found between the normal and the abnormal (atrophic as well as hypertrophic) kidneys is logically systematized Largest glomeruli are found in kidneys with high functional demands (in small normal or in hypertrophic abnormal kidneys), and smallest glomeruli are observed in large normal kidneys (with decreased functional demand per nephron) or in atrophic kidneys

The author would like to thank *Poul Faarup*, *Grete Ryo*, and *Sheila Sosnovec* for assistance

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ON THE SPECIFICITY OF THE G ABNORMALITY IN HUMAN MENINGOMAS STUDIED BY THE FLUORESCENCE TECHNIQUE

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The chromosomes were studied in eight human meningomas using the fluorescence technique Five tumours had a hypodiploid stemline with the loss of 1 No 22 two tumours had a pseudodiploid stemline with a structurally changed G22, and one tumour had a normal stemline but contained variant cells with the loss of one G22 The results were in accordance with previous preliminary observations and demonstrated that there was a specific G pattern in the human meningomas This G pattern was compared with that found in chronic myeloic leukaemia in man, and the similarities were proposed to be due to basic progressional mechanisms

The Ph 1 chromosome, in cases of chronic myeloid leukaemia (CML) in man, is still the only undisputable example of a consistent correlation between a specific chromosomal abnormality and a certain neoplastic condition The introduction of the fluorescence technique (*Caspersson et al* 1970), however, has created new possibilities for a rewarding search for such correlations A human tumour type of particular interest in this context is the meningomas as findings with ordinary staining methods (references in *Mark* 1971 a) have revealed an almost consistent loss of one or several G chromosomes in all tumours with karyotypic abnormalities The preliminary findings in 5 meningomas, studied by the fluorescence technique indicated that the pair G22 was primarily involved in both the numerical and structural variations of the small acrocentrics (*Mark et al* 1972) The results were very similar to those obtained by *Zankl* & *Zang* (1972) in a series also consisting of 5 meningomas The suggestive specificity of the pattern in group G, indicated by these observations, prompted studies of further tumours, and the findings in 8 new cases will be the subject of the present report

MATERIAL AND METHODS

The clinico-pathological characteristics of the 8 meningomas, M20–M27, are given in Table 1 All tumours were histologically benign One of them, M21 had a spinal location, the others were all found intracranially Several primary cultures were established from each meningoma, as described in detail earlier (*Mark* 1970 b) The cultures were harvested 6–13 days later, after treatment with 0 2 µg colcemid per ml medium for 1–2 hours Several fixations were made from each tumour in order to obtain a sufficient number of wellspread meta

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phases with a state of contraction suitable for the identification of individual chromosome pairs The chromosome preparations were made by air or flamedrying and stained with quinacrine mustard as in the previous study (*Mark et al* 1972) The equipment for the fluorescence microscope was also the same as in the earlier work The fluorescence karyotypes were determined in 18 or more cells in all meningomas, using magnified photos As in the previous work, the findings in group G were also checked in many additional cells, directly in the microscope Several slides from each tumour were stained with Giemsa for 5–10 minutes, and the chromosomes were counted in 50–80 cells This was done to determine the stemline (S) number but it was also a precaution taken to exclude the possibility that the sample studied by fluorescence was not representative for the tumour cell population The nomenclature for the marker chromosomes is the same as in *Mark* (1969)

RESULTS

The chromosome counts are shown in Table 1 All meningomas had their S in the hypodiploid diploid region Hyperdiploid counts were rare, the spread usually being hypomodal One or more polyploid cells were sometimes found in the 2S zone

Tables 2–7 give the fluorescence karyotypes observed in M20, M21, M23, M24, M25 and M26, respectively These results were recorded as differences relative to the normal male or female karyotype The observations in each meningoma are discussed below

M20 (Table 2) This tumour had a hypodiploid stemline, S=43, which was characterized by the loss of one member of the pairs A1, C6, C7, C11 and G22 and the gain of 1 t marker and 1 ring chromosome (*Figure 1 a–d*) The size of the t marker was always similar to that of an A1 chromosome The long arm of the t marker displayed two bright band complexes, separated by a narrow dark zone The proximal complex was indistinguishable from that of the shorter arm

TABLE 1 *Clinico Pathological Characteristics of the Material and Chromosome Counts*

Tumour M no	Sex	Age	Chromosome counts 40	41	42	43	44	45	46	47	48	84	92	Total cells	Histological type
M20	♀	36	2	3	11	31	8	2	1	–	–	2	–	60	Angioblastic
M21	♀	43	–	–		1	11	34	4	–	–	–	–	50	Angioblastic
M22	♀	67	–		–	1	2	44	3	–		–	–	50	Syncytial
M23	♂	68		–	3	4	5	34	4	–	–		–	50	Syncytial
M24	♀	49	1	3	4	3	8	21	9	–	1	–	–	50	Transitional
M25	♀	60		–	–	2	9	19	44	2	2	–	2	80	Transitional
M26	♀	47	–		1	3	2	9	54	–			1	70	Fibroblastic
M27	♀	55	–		–		1	7	42	–	–	–	–	50	Syncytial

TABLE 2 *Karyotypes of 20 Cells in M20*

Chromosome number	Affected chromosome pairs 1	2	6	7	9	11	19	22	Markers t	ring	Number of cells
40	—1		—1	—1	—1	—1	—1	—1	+1		1
41	—1		—1	—1	—1	—1	—1	—1	+1	+1	1
42	—1	—1	—1	—1		—1		—1	+1	+1	1
42	—1		—1	—1		—1	—1	—1	+1	+1	2
S–43	—1		—1	—1		—1		—1	+1	+1	12
44	—1		—1	—1				—1	+1	+1	1
44	—1		—1	—1		—1		—1	+1	+2	1
45	—1							—1	+1		1

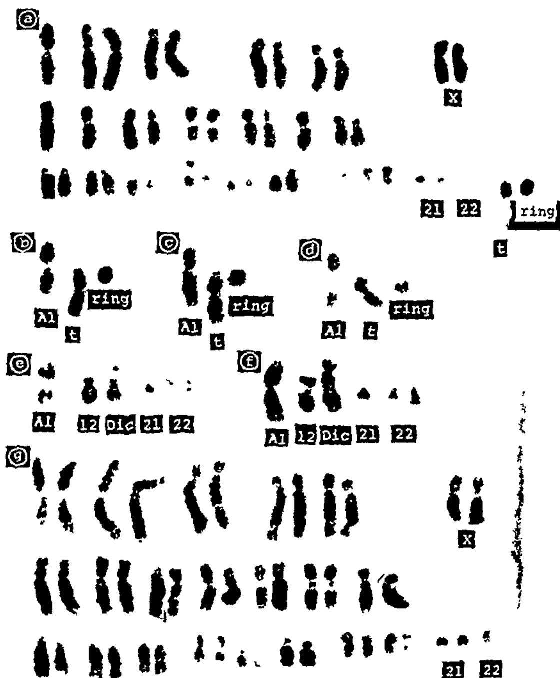


Fig 1 Complete (a and g) and condensed (b–f) karyotypes of *a–d*, S-cells in M20, *e*, *f*, variant cells in M21 and *g* S-cell in M22 × 1400

TABLE 3 *Karyotypes of 27 Cells in M21*

Chromosome number	8	Affected chr pairs 12	21	22	Markers dicentric	Number of cells
44			—1	—1		1
44	—1			—1		3
44	—1	—1	—1		+1	1
45		—1	—1		+1	6
S–45				—1		14
45		—1				1
46						1

of an A1, and the distal complex from that of the longer arm of an A1 The short arm of the t marker had a size and a fluorescence intensity as that of the long arm of a G22, but a definitive identification was not possible Thus it was obvious that both arms of the lost A1 chromosome had contributed to the formation of the long arm of the t marker The short arm of the marker was possibly derived from the long arm of the lost G22 This explanation was also supported by the occurrence of a variant cell, with 2n−45 having lost 1 A1 and 1 G22 and having gained just 1 t marker If the proposed trans location initially led to the formation of a dicentric this marker would be liable to the further structural rearrangments necessary for the appearance of the t marker, and the dicentric would also represent a natural source for the appearance of a ring chromo some The size of the latter marker was approximately two thirds of that of an A1 chromosome The bright fluorescence of the ring chromosome, except in its centromeric region, fitted with an origin from one or both arms of an A1 The losses among the other chromosome pairs could not be connected with the formation of the markers The pat tern in these groups as that of A1, G22 and markers, however, demonstrated very clearly the close relation between all elements of the tumour cell population, i e its clonal evolu tion and the fairly high frequency of deviat ing cells displayed that variant cells are com mon in, at least some, meningomas

M21 (Table 3), *M22*, *M23* (Table 4), and *M24* (Table 5) All these 4 hypodiploid tumours with S=45, had a stemline showing the classical karyotype of meningomas name ly the loss of 1 G chromosome In all stem lines this monosomy G concerned pair G22 One to four cells with a normal, diploid karyotype were seen in all tumours This pat tern of the other variant cells was as follows

TABLE 4 *Karyotypes of 20 Cells in M23*

Chromosome number	8	14	15	Affected chromosome pairs 17	20	21	22	Number of cells
42				—2	—1		—1	1
42	—1	—1	—1				—1	1
43			—1	—1			—1	1
43	—1					—1	—1	1
43	—1				—1		—1	1
44			—1				—1	1
44	—1						—1	1
45	—1							1
S=45							—1	10
46								2

TABLE 5 *Karyotypes of 25 Cells in M24*

Chromosome number	Affected chromosome pairs 8	9	10	15	21	22	XX	Number of cells
40	−1	−1		−1	−1	−1	−1	1
41	−1			−1	−1	−1	−1	1
41	−1		−1	−1		−1	−1	1
42		−1			−1	−1	−1	1
42	−2					−1	−1	1
43	−1				−1	−1		1
44						−1	−1	1
44	−1						−1	1
44	−1					−1		1
S−45						−1		11
46								4
48		+1				+1		1

In *M21*, some variant cells differed from the S cells by the loss of 1 G21 or 1 C8 A deviating and larger subgroup showed no loss of 1 G22 but instead of it there was a loss of 1 G21, 1 C12 and sometimes also of 1 C8 and the gain of 1 dicentric marker The latter was obviously formed by a translocation of the lost G21 onto the short arm of the missing C12 (Figure 1 e, f) The single cell having lost only 1 C12, was probably related to this subgroup with the dicentric marker

In *M22* 16 of the analysed cells had the S karyotype (Figure 1 g), and 1 cell showed no deviation from the normal The only other variant cell observed, with 2n=44, differed from the S cells by the loss of 1 E17

In *M23*, the loss of 1 C8 was the most frequent deviation among the aneuploid variant cells, usually together with a loss of 1 G22 (Figure 2 a) The other abnormalities observed were always losses, and they were distributed on 5 chromosome pairs D14, D15, E17, F20 and G21, with a slight preference for the pair D15

In *M24*, in addition to the loss of 1 G22 (Figure 2 b), the loss of 1–2 C8 chromosomes was also the most frequent deviation, together with the loss of 1 X chromosome The other losses engaged 4 pairs, C9, C10, D15 and G21, with a slight preference for the last two pairs

M25 (Table 6) This meningoma had a pseudodiploid stemline characterized by the loss of 1 G22 and the gain of 1 M marker (Figure 2 c f) The marker was somewhat smaller than a chromosome G22 the chroma

TABLE 6 *Karyotypes of 31 Cells in M25*

Chromosome number	Affected chr pairs 8	9	22	Markers M	t	Number of cells
44		−1	−1			1
44	−1	−1	−1	+1		1
44		−1	−2	+1		1
45	−1		−1	+1		2
45			−2	+1		2
45			−1			5
S−46			−1	+1		14
46						4
47		−1	−1	+1	+2	1

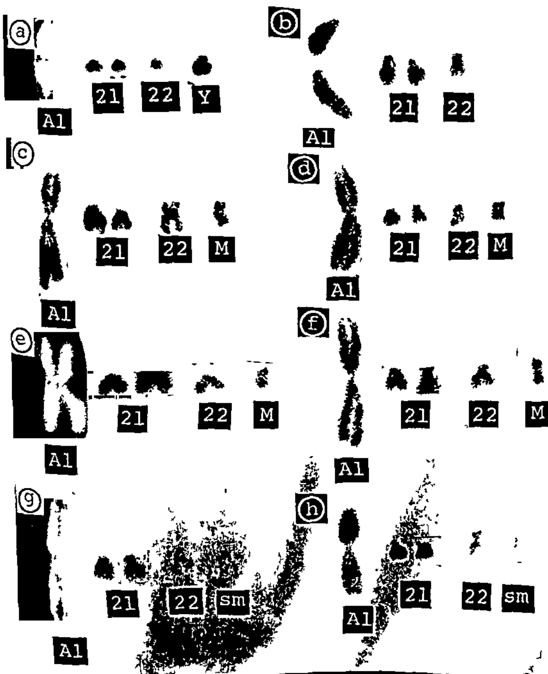


Fig 2 Condensed karyotypes of S-cells in *a*, M23, *b*, M24, *c f*, M25, and *g, h*, M26 × 2800

TABLE 7 *Karyotypes of 19 Cells in M26*

Chromosome number	Affected chromosome pairs 8	15	17	20	22	XX	Markers sm	Number of cells
43	—1	—1	—1					1
43			—2		—1	—1	+1	1
45				—1	—1		+1	1
45		—1			—1		+1	1
S=46					—1		+1	9
46								6

tids of its arms were often very close to each other, and satellites could be demonstrated on both arms in several cells (Figure 2 f) The morphological features, as the fluorescence intensity, of the small marker, fitted well with the idea that the M marker was an isochromosome of the short arm of the lost G22 The long arm of the missing G22, however, could not be traced to any other chromosome and it was probably lost The only other markers observed were found in the single hyperdiploid cell, with 2n=47, both of them were somewhat longer than the G chromosomes and displayed two bright bands in the middle of the long arm Their origin could not be traced As in previous meningomas, the variant cells were closely related to the S cells, and the deviations were concentrated to a few chromosome pairs namely C8 and C9, in addition to G22 and the group markers This makes it unlikely that the variation was an artifact caused by breakage of cells

M26 (Table 7) This meningoma had also a pseudodiploid S, which displayed the loss of 1 G22 and the gain of 1 sm marker The marker represented obviously a deleted chromosome G22 which had lost, approximately the distal third of the long arm (Figure 2 g h) In addition to the S, there was a subgroup of cells with a normal, diploid karyotype, and another subgroup with a loss of 1 G22 The remaining variant cell showed losses concentrated to 3 chromosome pairs, C8, D15 and E17

M27 The last meningoma had a normal, diploid stemline, no anomalies in group G being observed in any of the 16 S cells analysed In view of the findings in the other meningomas studied by the fluorescence technique, it was of interest that the 2 variant cells analysed, both with 2n=45, had lost 1 *G22*

DISCUSSION

The present series of 8 meningomas demonstrates a selective involvement of the chromosomes in group G Accordingly, 5 stemlines show the loss of 1 entire G22 (M20–M24), 2 stemlines are characterized by the loss of a part of 1 G22 (M25, M26), and 1 tumour, with a normal diploid S, has variant cells with the loss of 1 *G22 (M27)* This pattern in group G accords with the observations in 2 previous series of meningomas, each one consisting of 5 cases (*Zankl & Zang* 1972, *Mark et al* 1972) If these data are pooled with those of the present series, the G pattern in the whole material of 18 meningomas would be as follows 12 tumours with the loss of 1 entire G22, 3 tumours with the loss of parts of 1 G22, 2 tumours with a normal S but one of them having variant cells with a loss of 1 G22, and 1 tumour with a heteroploid S without any abnormality in group G but, instead of it, the loss of 1 C8 Thus, it can be regarded as an established fact that in human meningomas, there is a specific G pattern, the primary changes among the small acrocentrics being virtually confined to the pair G22 This specificity approaches that found in CML in man but there are several obvious differences In meningomas there is predominantly a numerical G variation, probably of non-disjunc-

tional origin, leading to the loss of an entire chromosome G22 Several types of rearrangements are on record among the few meningomas with a structural G variation In spite of this, a common denominator can be discerned for all types of G variation in meningomas namely the loss of approximately the distal third of the long arm of 1 G22 This loss could well be the initial change in all meningomas, usually causing mitotic disturbances and the loss of the deleted G22 but in a few cases instead leading to the development of a marker Such a small structural abnormality of one G22 would easily pass unnoticed in metaphases studied by ordinary staining methods (cf *Mark* 1970 b) This applies also to the fluorescence technique if the deletion is restricted to a few variant cells in tumours still having their original, normal diploid S

The implications of the specific G pattern in human meningomas, however, are controversial In CML in man the loss of the distal part of the long arm of 1 G22 is connected with an unlimited cell proliferation of the affected cells and their progeny, i e a malignant disease In the meningomas, however, whether there is a similar deletion of 1 G22, a different change of 1 G22, or the loss of 1 entire G22, the chromosomal abnormality is connected with the development of a benign tumour, which shows little tendency to a malignant transformation This discrepancy does not rule out the possibility that a growth regulating gene complex might be located in the distal part of the long arm of G22 (*Zankl* & *Zang* 1971), but the outcome of such a deletion is apparently influenced by other factors Among these are especially to be mentioned (1) the histogenetical nature of the cells subjected to neoplastic conversion, (2) the tumour inducing agent, and (3) also changes in other chromosomes than G22

(1) This factor was discussed in connection with the similar pattern of ploidy observed in malignant neurogenic tumours in adults and in children (*Mark* 1971 b) As regards meningomas and CML, a similar chromosomal deviation might have different evolutionary consequences due to the dissimilarities between the blood cells and the meningeal cells in their ontogenic derivation and differentiation

(2) The possible influence of the tumour inducing agent cannot be evaluated It is well known, however, that many oncogenic agents are selective as to the cell types that they can transform and recent observations in animal tumours strongly indicate that different oncogenic agents induce different, chromosomal patterns (*Mark* 1970 a, *Mitelman et al* 1972) It seems unlikely that the same agent should be involved in meningomas and CML

(3) Most stemlines in the meningomas showed no gross morphological deviations other than those affecting G22 This does

TABLE 8 *The Mean Distribution (%) of Chromosomal Deviations on Different Chromosome Groups of All Cells Analysed in M15 M27*

0–1 %		1 1 7 %		7 1 100 %			
B5	0/0	E16	1 6/II	C11	7 2/II	Markers	32 8/VI
B4	0 3/I	F20	2 2/IV	C6	7 3/I		
C10	0 3/I	X	2 6/II	C7	7 6/II		
D14	0 4/I	D15	2 9/III	E17	8 3/V		
E18	0 4/I	Y	3 6/I	C1.	9 6/II		
A3	0 6/II	C9	4 4/V	A1	13 6/III		
A2	1 0/III	G21	6 2/V	C8	24 3/IX		
D13	1 0/III	F19	6 7/IV	G22	65 6/XII		

Arabic numerals %
Roman numerals = number of tumours with engagement of a certain chromosome group

not exclude, however, the presence of sub microscopic changes These changes may well predispose (*Nichols* 1966) to the abnormalities found in variant cells and to the additional deviations in stemlines showing chromosome changes other than that affecting G22 To evaluate this pattern, all karyotyped cells in M15–M27 were used to calculate the mean frequency of involvement of each chromosome pair in each tumour, and these data were used to determine the mean values for the whole material of 13 meningomas The 5 meningomas of *Zankl* & *Zang* (1972) were not included, since all data were not available from them Table 8 shows the results, the ordinary chromosome types being listed in the order of increasing frequency of involvement, the number of meningomas manifesting an involvement of a particular chromosome is also shown in the table by Roman numerals The only consistent deviation concerns pair G22 The changes in other chromosome pairs, however, are not randomly distributed Thus, group C is especially often involved and there is an obvious preference of one particular pair, namely C8 Other features of interest are the common engagement of the pairs E17 and A1 and also the low frequencies found for G21 and the Y chromosome This pattern of variation in the meningomas with several preferential trends is different from that observed in CML In this disease additional changes are usually few and so far show no consistent pattern (*Sandberg* & *Hossfeld* 1970) This difference between meningomas and CML strengthens the impression that the similarities of the deviation in group G are gross morphological features probably related to basic progressional mechanisms

The present findings by fluorescence microscopy of 13 meningomas have confirmed that structural abnormalities are fairly frequent and the results also showed that variant cells are common in many meningomas The opposite conclusions have been emphasized by other investigators (*Zang* & *Singer* 1970 *Zankl* & *Zang* 1971) These questions and related ones will be discussed in a later report dealing with observations in the total material consisting of 50 tumours

ACKNOWLEDGEMENT

The investigation was supported by grants from the Swedish Cancer Society, the University of Lund and by grants from John and Augusta Persson s foundation for Medical Research

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Acta path microbiol scand Section A 80, 821–826 1972

COMPARATIVE LIGHT AND ELECTRON MICROSCOPICAL STUDIES OF DECAYING THYMIC LYMPHOID CELLS

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Normal thymic lymphoid cell suspensions were studied in the electron microscope The suspensions contained cells (6–23 per cent) in various degrees of lytic degeneration with swelling and disintegration of both nucleus and cytoplasm Only a minority of the degenerating cells showed pyknotic changes and these cells had always a well preserved cytoplasm The percentage of degenerating cells was compared with the percentage of cells stained in the nigrosin dye exclusion test A close relationship between these two values was found, suggesting that supravital staining primarily affects cells in lytic cell degeneration It appears that vital stains only affect already degenerating cells with a subsequent increase of a preexisting intracellular edema This suggestion was corroborated by the finding that addition of nigrosin dye to thymic cell suspensions does not depress aerobic metabolism

The dye exclusion test is currently used to distinguish between living and dead cells Stain diffuses into the cytoplasm and nucleus of non viable cells while viable cells remain unstained Functional impairment of the stainable cells has been described in numerous works and all authors agree that the metabolism of these cells is irreversibly changed to such a degree that they can be considered dead or dying (*Phillips & Terryberry* 1957, *Eaton et al* 1959, *Kaltenbach et al* 1958, *Holmberg* 1961) In spite of these findings decaying lymphoid cells cannot be distinguished morphologically from viable cells by means of conventional light microscopy

The present investigation was undertaken in order to establish whether the stainable cells show ultrastructural signs of degeneration which could explain the uptake of vital stains It was further considered of interest to describe the ultrastructural changes which follow addition of a vital stain (nigrosin) to unfixed lymphoid cell suspensions In order to study the possible toxic action of nigrosin on lymphoid cells, oxygen consumption was measured after addition of nigrosin

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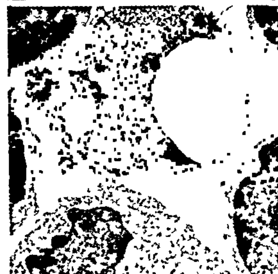
Requests for reprints should be addressed to Dr *M H Claesson* The Institute of Medical Anatomy A University of Copenhagen 71 Rådmandsgade, DK 2200 Copenhagen N, Denmark

MATERIAL AND METHODS

Suspensions of thymic lymphocytes were obtained from 1 and 5–6 months old Balb/C mice and nigrosin dye exclusion performed on suspensions from individual mice as previously described (*Claesson* 1969) Aliquots of each cell suspension were prepared for electron microscopy The cells were centrifuged and the cell pellet fixed in 2 5 per cent glutar aldehyde for half an hour washed and postfixed in 1 per cent OsO_4 for 1 hour After washing the pellet was dehydrated in graded alcohols followed by embedding in Epon 812 The preparations were cut on an LKB ultramicrotome and studied in a Hitachi electron microscope The quantitative electron microscopical studies were







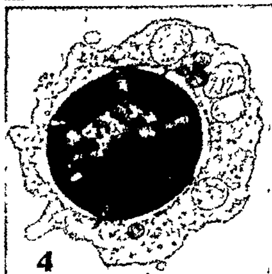


Fig 1 Early cell degeneration Dilated perinuclear cisterna, darkening of cytoplasm, and aggregation of ribosomes × 17 500

Fig 2 Advanced cell degeneration The nuclear material has broken through the nuclear *envelope* The cytoplasm is severely changed × 12 000

Fig 3 Advanced cell degeneration Cytoplasmic organelles and nuclear material in the intercellular space × 11 500

Fig 4 Nuclear pyknosis with well preserved cytoplasm × 19 000

ased on survey micrographs with a primary magnification of 1000–2000 This allowed count ing of about 500 cells from each suspension Some of the suspensions were stained for 5 minutes with 0 1 per cent solution of nigrosin in physiological saline prior to the preparation for electron microscopy

The aerobic glycolysis was measured in a conventional Warburg apparatus The thymocytes were washed in PBS with 0 15 per cent glucose and resuspended (25 × 10[6] cells per ml) The cell suspensions were incubated at 38°C either in equal parts of a 0 1 per cent nigrosin solution in physiological saline or in equal parts of physiological saline Oxygen consumption was measured with intervals of 15 minutes during a period of 2 hours

RESULTS

Ultrastructure of Thymic Cell Suspensions

Normal cells The ultrastructure of thymic cells corresponds to previous descriptions (*Clawson et al* 1967) The amount of cytoplasm was comparatively small, often it was only a narrow rim surrounding the nucleus Ribosomes and mitochondria were seen in all cells the latter being by far more conspicuous in the larger cells Small irregular microvilli were seen projecting from the cell surface About 90 per cent of the cells had an average diameter less than 7 microns

Degenerating cells Varying degrees of cellular degeneration were obvious in all cell suspensions An irregular widening of the perinuclear cisterna together with a shortening and a decrease of the number of microvilli indicated a mild degree of cell degeneration In many cells a diffuse darkening of the cytoplasm was also seen and often the ribosomes were gathered in groups (Fig 1) In more advanced stages of degeneration the nuclear material was disintegrated and scattered all over the nuclear area When degeneration had progressed further the nuclear membrane was often torn so that nuclear material which appeared to be thinned out could be found in the the cytoplasm (Fig 2) The plasma membrane was often totally disrupted and the organelles were seen in the extracellular space (Fig 3) Occasionally pyknotic cells were seen but in contrast to the degenerating cells mentioned above they had always a well preserved cytoplasm (Fig 4)

The percentage of degenerative cells was determined for each animal and compared with the results of the nigrosin dye exclusion test The percentage was calculated from survey micrographs (Fig 5) Table 1 shows the close quantitative relationship between these two values A certain discrepancy between the results obtained with the two methods was only noted in two animals (no 1149 and 1153)

Thymocytes Stained with Nigrosin

Generally speaking the ultrastructure of degenerating cells of the nigrosin treated suspensions was characterized by a cell damage more heavy than that found in the unstained suspensions These damaged cells were enormously dilated with cell diameters averaging 30–40 microns The cytoplasm was completely destroyed and the nuclear membrane disrupted In spite of the rupture of the membrane nuclear material could generally be found evenly distributed within the confines of the former nuclear area (Figs 6–7)

Oxygen Consumption

Incubation in nigrosin did not depress the oxygen uptake of thymic lymphoid cells as compared with non stained cells During the incubation period (2 hours) the oxygen consumption averaged 10 microMol/10[9] cells/hour

DISCUSSION

Under normal conditions unfixed as well as fixed preparations of lymphoid cell suspensions contain decaying cells as judged by nigrosin dye exclusion and ultrastructural investigations The vast majority of cells with ultrastructural signs of decay were in various stages of lytic cell degeneration which affected both nucleus and cytoplasm The same type of lymphoid cell degeneration has also been described after *in vitro* treatment of lymphocytes with heterologous antiserum (*Land et al* 1968), isologous antiserum +

Fig 5 Survey micrograph of thymic cell suspension Degenerative cells are labelled × 5 000

Figs 6 and 7 Varying degrees of cell swelling and damage due to nigrosin diffusion × 13 000 and × 11 500

complement (*Walford et al* 1966, *Claesson et al* 1971) and after treatment with various lymphocytotoxic agents such as x-rays and cortisone (*Trowell* 1966) indicating a non specific action of these agents A non-specific action of antiserum + complement was also considered a possibility by *Claesson et al* (1971) A few cells showed pyknosis (see

below) This kind of degeneration is obviously confined to the nucleus since the cytoplasm appeared normal in most cases In this connection it should be mentioned that the pyknotic cells show a considerable resistance to vital dyes in the dye exclusion test (*Pappenheimer* 1917, *Claesson* & *Ropke* 1969)

TABLE 1 *The Percentage of Decaying Thymic Lymphoid Cells as Judged by Dye Exclusion Test and Electron Microscopic Examinations Respectively*

Animal no.	Age months	Decaying thymocytes: Dye exclusion stained cells	Decaying thymocytes: Electron microscopy degenerative cells
1146	1	7 5%	9 3%
1149	1	12 6%	6 0%
1150	1	8 2%	8 2%
1151	1	7 3%	6 5%
1152	1	10 6%	13 0%
	Mean	9 2%	8 6%
1145	5	17 8%	14 3%
1153	7	15 6%	8 7%
1154	6	17 5%	17 0%
1155	5½	12 1%	10 3%
1156	5	22 1%	23 0%
	Mean	17 0%	14 7%

To day it is generally accepted that the majority of cells produced in the thymus decay *in situ* while a minority leave the organ (*Everett* & *Tylor* 1970) It must be admitted however that the number of pyknotic thymocytes seen under normal conditions is too low to explain such an extensive degree of cell death (*Nakamura* & *Metcalf* 1961 *Sainte Marie* & *Leblond* 1964 *Michalke et al* 1969) For this reason *Metcalf* & *Brumby* (1966) proposed another theory of thymocyte degeneration the thymocyte explosion which being invisible cannot be observed in the light microscope (*Metcalf* 1966) The ultrastructural changes seen in degenerating thymocytes are in line with the suggestion advanced by *Metcalf* & *Brumby*

The fact that the same amount of decaying cells are seen in unfixed and fixed cell suspensions (see Table 1) furthermore suggests that supra vital staining affects cells in various stages of lytic cell degeneration while pyknosis can be held responsible only for a minor degree of thymocyte decay

The age dependent variations of thymic lymphoid cell decay shown by the use of the dye exclusion test (*Claesson* 1969) was confirmed in the present study where the mean values of the two age groups differed significantly ($p < 0\ 05$, rank sum test) The difference between values obtained with electron microscopical quantitation of degenerative cells differed at a lower level of significance ($p < 0\ 1$, rank sum test)

If electron micrographs of nigrosin stained and non stained cell suspensions are compared it seems safe to conclude that nigrosin alters already decaying cells with a resulting increase of cellular destruction Normal cells —on the other hand—do not seem to become damaged by the addition of nigrosin as the oxygen uptake was not depressed in stained cell suspensions

It may be concluded that normal thymocyte suspensions show varying degrees of cellular degeneration in the electron microscope and that the number of cells with ultrastructural signs of degeneration equal the number of cells stained in the dye exclusion test The decaying cells can be considered to be in various stages of lytic cell degeneration Oxygen consumption of nigrosin incubated thymic cell suspensions as well as ultrastructural studies indicate that nigrosin only attacks already degenerating cells

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Acta path. microbiol scand Section A 80, 827–834, 1972

RENAL TRANSPLANTATION IN RABBITS

IX An Immunological Study of the Allograft Reaction in Rabbits Pre-sensitized by Donor Kidney Homogenate

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IgG deposition was studied in 35 biopsy specimens from 15 renal allografts into rabbits pre sensitized by donor kidney homogenate Glomerular and vascular fluorescence was observed in specimens removed two hours after transplantation Glomerular IgG deposits were found in eight and vascular IgG deposits in nine allografts The IgG deposits were not correlated to histological alterations in glomeruli or intrarenal blood vessels Lymphocytotoxic antibodies were demonstrable from three weeks after the first injection of kidney homogenate and lymphocytotoxic antibodies developed in all rabbits after 6–10 weeks The presence of lymphocytotoxic antibodies in the serum of kidney recipients prior to kidney transplantation and IgG deposition in glomeruli or intrarenal blood vessels were not associated with severe types of allograft reactions

In the study presented here histological alterations in renal allografts from rabbits pre-sensitized by donor kidney homogenate were related to antibody (IgG) deposits in the grafts and to the presence of lymphocytotoxic antibodies in the serum of recipients prior to kidney transplantation

The study is a continuation of similar investigations in rabbits pre sensitized by multiple skin grafts (*Lund & Sommer Hansen* VII 1972 *Ahrons & Lund* VIII 1972)

MATERIAL AND METHODS

Animals Fifteen donors and 15 recipients were chosen from different strains of outbred New Zealand white rabbits and brown lop eared rabbits

Sensitization Prior to kidney transplantation each recipient was sensitized by five weekly subcutaneous injections of donor kidney homogenate Freund s complete adjuvant was added to the first two aliquots of homogenate Seven rabbits had no lymphocytotoxic antibodies six weeks after the first injection of homogenate and were given a further dose of homogenate intraperitoneally (*Lund & Myhre Jensen* 1971)

Serological investigation The titre of lymphocytotoxic antibodies was determined before and at 2 10 weeks after the first injection of homogenate The last determination was performed 1 3 days before kidney transplantation The details of these procedures were reported by *Ahrons & Lund* (1972)

Kidney transplantation The surgical technique and methods used for the preparation of specimens for histological study and immunofluorescence microscopy were as described by *Lund* (1970), *Lund & Myhre Jensen* II (1970) and *Lund & Sommer Hansen* VI (1972)

Immunofluorescent examination For the details of the technique, controls and photographic recording, see *Lund & Sommer Hansen VI* (1972)

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TABLE 1 *IgG Deposits and Histological Alterations in 15 Renal Allografts from Rabbits Sensitized by*

Biopsy specimens removed after	Rabbit No	Lymphocytes fluores	Lymphocytes	Glomerular fluores	Prolif glomerulitis	Exudat glomerulitis
2–6 hours	265	—	—	+	—	—
	283	—	—	—	—	—
	285	—	—	+	—	—
	289	—	—	—	—	—
	291′	—	—	—	—	—
1 day	255	—	—	—	—	—
	259	—	—	—	+ + +	—
	261′	—	—	—	—	—
	263	—	—	+	—	—
	271′	—	—	+	—	—
	277	+	+	—	+ +	—
	279	—	—	—	—	—
	281′	+	+	—	—	—
	283	—	—	+	—	—
	285	—	—	—	—	—
	287	+	+ +	—	+	—
	289	—	—	—	—	—
	291′	+	+	+	+	+
2 days	259	—	—	—	+ +	—
	261	—	—	—	—	—
	263	+	+ +	+	+ +	—
	273	—	—	—	—	—
	283	—	—	+	—	—
	285	—	—	—	—	—
	291	+	+ +	+	+	—
3 days	259	—	—	—	—	—
	271′	—	—	—	—	—
	279	+	+ +	+	+ +	—
	281	—	—	—	—	—
	285	—	—	—	—	—
4 days	271	—	—	+	—	—
	283	—	+	+	+	—
	285	—	—	—	—	—
	291	+	+ +	—	+ +	—
6 days	277	+	+ + +	+	+ + +	—

Reactions in brackets indicate partial necrosis probably of non immunological origin
Biopsies are marked by

RESULTS

The results of the immunofluorescent study, histological examination and the pre operative titre of lymphocytotoxic antibodies are summarized in Table 1

Histological examination of the allografts from rabbits pre-sensitized by donor kidney

Donor Kidney Homogenate in Relation to Pre Operative Titre of Lymphocytotoxic Antibodies

Vascular fluorescence	Endarteritis	Vasculitis	Micro-thrombosis	Necrosis	Pre-op cytotoxic antibody titre
+	—	—	—	—	≥ 8
—	—	—	—	—	≥ 8
—	—	—	—	—	≥ 8
—	—	—	—	—	4
—	—	—	—	—	4
—	—	—	—	—	≥ 8
—	—	—	—	—	4
—	—	—	—	(+ + +)	1
+	—	—	—	—	1
+	—	—	—	—	≥ 8
+	—	—	—	—	≥ 8
—	—	—	—	—	4
—	—	—	—	—	4
—	—	—	—	—	≥ 8
+	—	—	+ +	—	≥ 8
—	—	—	—	—	4
—	—	—	—	—	4
—	—	—	—	—	4
—	—	—	—	+	4
+	—	—	—	+ +	1
+	—	—	—	—	1
—	—	—	+ + +	+ + +	≥ 8
—	—	—	—	—	≥ 8
—	—	—	+ + +	+ + +	≥ 8
+	—	—	—	—	4
—	—	—	—	+ + +	4
—	—	—		—	≥ 8
+	—	—		—	4
+	—	—		(+ + +)	4
—	—	—	+ +	+ + +	≥ 8
+	—	—	—	—	≥ 8
—	—	—		+	≥ 8
—	—	—	+ + +	+ + + +	≥ 8
+	—	+	+	+	4
—	—	—	+	—	≥ 8

homogenate was reported in detail in a previous paper (*Lund & Myhre Jensen* V, 1971) Fifteen of the original 18 allografts were available for immunofluorescent study

Briefly the most important histological altera tions were Seven of the 15 allografts had prolifera tive glomerulit - and one exudative glomerulitis

Vasculitis was seen in one allograft, while end arteritis was not observed in any of the grafts Seven allografts had perivascular lymphocyte in filtrations and two presented multiple glomerular microthrombosis and cortical necrosis Two other allografts showed partial necrosis of the cortex and medulla, probably of non immunological origin In four of the 15 renal allografts no lesions other

Key to semi quantitative light microscopic evaluation

	Perivascular lymphocyte infiltrates	Changes in glomeruli	Changes in blood vessels	Necrosis
+	Small	Less than 10 %	A few	Patchy cortical necrosis
+ +	Many or large	10–50 %	Many	Subcapsular zone
+ + +	Diffuse	More than 50 %	Almost all	Partial necrosis of cortex and medulla
+ + + +				Total necrosis

than interstitial oedema, hyperaemia or tubular lesions were found

Immunofluorescent Examination

Glomerular IgG deposits were observed in eight of the 15 allografts In two of these cases, fluorescence was seen in biopsy specimens obtained two and three hours after transplantation The deposits appeared as continuous linear fluorescence following the contours of the glomerular capillaries A granular pattern of fluorescence in the mesangial region was observed in two cases

Glomerular fluorescence was found in five of seven grafts with proliferative glomerulitis The fluorescence appeared earlier than proliferative glomerulitis in two cases, later than proliferative glomerulitis in one case, while simultaneous occurrence was seen in two cases One of the two cases with multiple glomerular microthrombosis and cortical necrosis showed glomerular fluorescence IgG

TABLE 2 *Development of Lymphocytotoxic Antibodies in Serum of Rabbits during Sensitization with Donor Kidney Homogenate*

Rabbit No	Titres of antibody before and at 2–10 weeks after the first injection of kidney homogenate Before	2	3	4	5	6	10
255	0 §	0 §	8 †	8 †	8 †	8 *	
259	0 §	0 §	0 †	0 †	0 †	4 *	
261	0 §	0 §	0 †	0 †	0 †	0 •	1 *
263	0 §	0 §	0 †	0 †	0 †	0 •	1 *
265	0 §	0 §	0 †	0 †	8 †	8 *	
271	0 §	0 §	8 †	8 †	0 †	0 •	8 *
273	0 §	0 §	0 †	0 †	0 †	8 *	
277	0 §	0 §	0 †	0 †	0 †	0 •	8 *
279	0 §	0 §	0 †	0 †	0 †	0 •	4 *
281	0 §	0 §	0 †	0 †	0 †	0 •	4 *
283	0 §	0 §	0 †	0 †	8 †	8 *	
285	0 §	0 §	8 †	8 †	1 †	8 *	
287	0 §	0 §	0 †	0 †	0 †	0 •	4 *
289	0 §	0 §	8 †	0 †	0 †	4 *	
291	0 §		8 †	8 †	0 †	4 *	

§ Subcutaneous injection of d[illegible]mogenate and Freund s complete adjuvant
† Subcutaneous injection of donor [illegible]ogenate
• Intraperitoneal injection of donor k[illegible]genate
* Kidney transplantation

deposits were also observed in two grafts without histological signs of rejection

In seven of the eight cases, glomerular fluorescence was accompanied by vascular fluorescence

Vascular IgG deposits were seen in small arteries and arterioles in nine of the 15 allografts In one of these vascular deposits were observed in a biopsy specimen obtained two hours after transplantation The deposits consisted of both fine and coarse granules in the media of the arteries and arterioles, whereas no intima or perivascular fluorescence was seen Only in one case was vascular fluorescence accompanied by vasculitis Vascular IgG deposits were found in one of the two allografts with multiple glomerular microthrombosis

Tubular fluorescence was found in nine cases as a granular pattern localized basally in the cytoplasm of the tubular cells No IgG deposits were observed in peritubular capillaries

Mononuclear cells with cytoplasmatic fluorescence were seen in six of the 15 allografts The number of mononuclear cells with fluorescence was smaller than the total number of infiltrating mononuclear cells The fluorescence was demonstrated both in small lymphocytes and in larger mononuclear cells

Controls Twenty two autografts and ten normal kidneys revealed no glomerular or vascular IgG deposits whereas fluorescence was recognized in tubuli and lymphocytes (*Lund & Sommer Hansen* VI, 1972)

Lymphocytotoxic Antibod es

The development of lymphocytotoxic antibodies during sensitization is illustrated in Table 2 Lymphocytotoxic antibodies could not be demonstrated before sensitization In five cases antibodies were found three weeks after the first injection of homogenate In seven cases no antibodies were observed after five injections of homogenate, and a further dose of homogenate was given intraperitoneally Three weeks later lymphocytotoxic antibodies were present in all rabbits

The titre of antibody was not studied in dilutions higher than 1 8 Of the rabbits nine had lymphocytotoxic antibodies active in the dilution 1 8 (or higher?), four in dilution 1 4, while two showed activity only in non diluted serum (both belonged to the group of rabbits without lymphocytotoxic antibodies in serum after five subcutaneous injections of homogenate) Only two of the sera reacted with lymphocytes from more than 20 per cent of the rabbits including those from the homogenate donor In one of the two rabbits with hyperacute type of allograft reaction (multiple glomerular microthrombosis and cortical necrosis) antibodies developed early (at three weeks), and a high titre of antibody was observed during the sensitization period (No 285) The antibodies represented the broadest spectrum seen in the study and reacted with the lymphocytes of eight other rabbits The second rabbit (No 273) with hyperacute type of reaction had a positive titre six weeks after the first injection of homogenate, and the serum reacted with the lymphocytes of the specific donor and one other rabbit

DISCUSSION

The presence of donor specific lymphocytotoxic antibodies in the serum of kidney recipients have been reported to be associated with early failure of kidney allografts in man (*Kissmeyer Nielsen et al* 1968, *Morris et al* 1968, *Terasaki* 1968, *Patel & Terasaki* 1969, *Hume et al* 1969 *Cochrum & Kountz* 1969, *Starzl et al* 1970)

In our experiments, however, donor specific lymphocytotoxic antibodies active in the dilution 1 4 or higher in 13 of 15 rabbits were not associated with a hyperacute type of reaction in more than two cases, even though 10 of the allografts had glomerular or vascular antibody (IgG) deposits

A correlation between glomerular antibody deposition and glomerular lesions could not be demonstrated in the present material Glomerular fluorescence was observed in five of the seven cases with proliferative glomerul

TABLE 3 *The Frequency of IgG Deposition in Renal Allografts from Non Sensitized Rabbits and from Rabbits Pre Sensitized by Donor Kidney Homogenate*

	Total No	Glomerular IgG deposits	Vascular IgG deposits	Fluorescence of infiltrating lymphocytes	Tubular fluorescence
Non sensitized recipients	20	0	4	14	12
Recipients sensitized by homogenate	14	5	7	6	7

The specimens were removed during the first 1 3 days after transplantation

		p (Fisher)	$p(X^2)$	$p(X^2)$	$p(X^2)$
		$p<0\,007$	$0\,10>p>0\,05$	$p<0\,05$	$0\,45>p>0\,40$

itis while the two remaining animals had no deposits, in another three glomerular fluorescence was observed without histological signs of glomerulitis

Compared with allografts from non sensitized rabbits glomerular and vascular fluorescence appeared earlier (two or three hours) in allografts from homogenate sensitized than in non sensitized animals (two or three days) (*Lund & Sommer Hansen* VI 1972)

The frequency of IgG deposition in renal allografts removed during the first 1 3 days after transplantation from non sensitized and homogenate sensitized rabbits is shown in Table 3 The limit of 1–3 days was chosen because severe types of allograft reactions appears during this period The same limit was used for comparison of IgG deposits in renal allografts from rabbits pre sensitized by multiple skin grafts and non sensitized animals (*Lund & Sommer Hansen* VII 1972) The frequency of glomerular IgG deposition was increased in the homogenate sensitized group ($p < 0\,007$) whereas that of vascular fluorescence was not significantly increased ($0\,10 > p > 0\,05$) There was no significant difference in the frequency of fluorescence in tubular cells and interstitial mononuclear cells in the two groups

Compared with renal allografts from rabbits pre sensitized by *multiple skin grafts* glomerular and vascular fluorescence appeared at the same time after transplantation (*Lund & Sommer Hansen* VII 1972) Almost the same frequency of glomerular and vascular fluorescence was seen in the two groups and lymphocytotoxic antibodies were observed prior to kidney transplantation in both groups However half of the 48 kidney allografts from rabbits pre sensitized by skin grafts showed a hyperacute type of reaction while only two of the 15 allografts in the homogenate sensitized group showed this pattern of reaction suggesting that the antibodies induced by skin grafts are different from those provoked by kidney homogenate

The antigenic effect of kidney homogenate also differs from that of pre sensitization by *kidney grafts* since *Klassen & Milgrom* (1969) reported that second set kidney grafts in rabbits suffered an accelerated type of rejection characterized by exudation of polymorphonuclear leucocytes in the glomeruli and intrarenal blood vessels A similar pattern was observed only in one of our rabbits (No 291)

Pretreatment of kidney recipients with *other donor tissues* (spleen liver bone marrow lymphocytes) has been reported to prolong the survival of kidney allografts due to enhancement or actively induced tolerance (*Owen et al* 1968 *Zimmerman et al* 1968 *Stuart et al* 1968 *Taguchi et al* 1968 *French & Batchelor* 1969 *Wilson et al* 1969 *Owen* 1970 *Ockner et al* 1970 *Holl Allen et al* 1970 *Zimmerman* 1971 *Taguchi et al*

1971 Wilson et al 1971) Since only two of our allografts showed a severe type of allograft reaction, it is possible that kidney homogenate has a similar effect However, our experiments were not designed to follow prolonged survival of the grafts, but to study severe reactions during the first few days after transplantation

The present experiments showed that donor specific lymphocytotoxic antibodies in the serum of kidney recipients prior to kidney transplantation and deposition of antibodies (IgG) in renal allografts were not correlated to severe types of allograft reactions

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The study was aided by grants from the *Danish Foundation for the Advancement of Medical Science and F L Smidth and Co A/S Foundation*

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Acta path microbiol scand Section A 80, 835 840 1972

METASTASES TO THE UTERUS

Five Cases Diagnosed on the Basis of Curettings

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Five cases of endometrial metastases from extrapelvic tumours are reported The diag nosis was based on curettings Four of the primary tumours affected the breast In the 3 cases where the histological specimens were obtained for revision the tumours proved to be of a special histological type resembling *épithélioma à cellules indépendantes et sécrétantes* The fifth primary tumour was in the stomach A common feature was the presence of mucus filled signet ring cells without extracellular mucus The endometrial metastases showed similar appearances, but were cellular unlike the breast cancers

Since it is important to bear in mind, from a diagnostic as well as therapeutic point of view, that a malignant tumour in the uterus need not be primary, it seems justified to publish 5 cases of metastases to the uterus from tumours of extrapelvic organs Such metastases were first described in 1878 (6), but they have rarely been mentioned in the literature and if so merely as reports of single cases or of very small series

In 1961 *Weingold & Boltuch* (15) collected 150 cases, mainly from Anglo Saxon literature, and added one case of their own In reviewing the literature we found another 15 cases (1, 2, 4, 5, 9 11 14) and 13 cases have been published later (8 10 12, 13) bringing the total up to 179

The majority have been diagnosed on the basis of curettings or cervical biopsies, whereas a few have been autopsy findings (1, 4)

The metastases have been localized to the endometrial or cervical mucosa with varying degrees of encroachment upon the myometrium or isolated in the myometrium (1, 4) Metastases in fibromyomas are on record (15)

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MATERIAL

Among 96 000 all round diagnostic microscopic examinations done for various hospitals we have diagnosed carcinomatous metastases to the endometrium in 4 cases on the basis of uterine curettings The fifth case was kindly supplied by our colleagues in the Department of Pathology of the Odense Hospital The site of the primary tumour was the breast in 4 cases and the stomach in one We have not included intrapelvic tumours with direct propagation or metastasization to the uterus

Case Reports

Case 1 (64495) A 49 year-old woman admitted in December 1966 with a 2½ cm tumour in the right breast No swelling of the regional lymph nodes Microscopic examination revealed carcinoma and the patient was treated by mastectomy and postoperative X ray irradiation In January 1970 she was re admitted as a case of menopausal metrorrhagia Up to April 1969 her menstrual periods had been regular 7days/28 days There after menostasis until the end of November 1969 when she began to have daily oozing bleeding interrupted by one period reminiscent of menstruation towards the end of December 1969

The uterus was found to be situated in the midline, anteflexed, of normal shape and size Microscopic examination of curettings revealed solid metastatic carcinoma of the endometrium and myometrium Laparotomy disclosed ascites and small white spots on the small intestinal serosa, interpreted as carcinosis, but no biopsy was taken No palpable metastases in the liver Supra vaginal hysterectomy by the method of Chrobak and bilateral salpingo-oophorectomy were per formed The removed uterus was 7 × 7 × 6 cm In one wall the mucosa was slightly nodular, and at this site the wall was somewhat thickened and perhaps rather firm, but without gross focal changes The adnexa showed no gross abnormalities apart from a slightly thickened Fallopian tube and a couple of small cysts in one ovary Microscopy revealed tumour cells, like those found in the endometrial curettings, in remnants of the endometrium In all the sections of the myometrium there was diffuse neoplastic infiltration without any well defined nodes The same findings were made in the tubes and ovaries In the tubes there was especially infiltration of the muscularis and subserosa Postoperatively the patient was treated by Endoxan® and X rays During the stay in hospital she did not exhibit any signs of recurrence of the primary tumour and had no palpable axillary nodes Mammography of the left breast and chest radiography were normal

She was re admitted in August 1971 with pain in the back X ray examination showed wide spread metastases in the spine

Case2 (72280) A 63 year-old woman admitted in January 1966 with a 4 cm tumour of the left breast Menopause 13 years before admission Since then neither vaginal bleeding nor discharge Apart from an enlarged lymph node in the left axilla no signs of dissemination Right breast normal After left sided mastectomy microscopic examination showed carcinoma The patient had postoperative irradiation After the operation she regularly attended out patient follow up the last time in May 1971 without any signs of recurrence being detected In June 1971 she began to have increasing pollakisuria and at the same time her abdomen increased in circumference At re admission in August 1971 she had severe ascites Gynaecological examination showed the portio and anterior vaginal wall to be filled with an infiltrating mass spreading through the entire true pelvis Chest radiography showed no signs of metastases Diagnostic curettage revealed metastatic carcinoma of the endometrium Thereafter the patient was transferred to a radium centre where she was treated with Prednisolone and radioactive gold When last seen in November 1971 she was feeling surprisingly fit and looked well

Case 3 (P 9297) A 59 year old woman admitted in August 1959 with a tumour of the right breast. Retraction of the right nipple Right sided mastectomy was done with removal of the axillary nodes and the lateral part of the pectoralis major Histological examination showed carcinoma Postoperative X ray irradiation In June 1963 the patient was re admitted because of repeated vomiting through six months and epigastric pain Three months prior to this admission X rays of the chest had shown signs of metastases to the spine and ribs Menopause 13 years ago One week before admission minor vaginal bleeding had occurred. Gynaecological examination revealed a polyp, 0 5 × 4 cm in the os and firm infiltration in the true pelvis Microscopic examination of curettings from a small irregular cavity revealed metastases of carcinoma to the endometrium Palliative irradiation was administered In June 1965 the patient was re admitted with severe dyspnoea X ray examination showed a large pleural effusion on the right and multiple metastases to numerous bones of the thoracic cage The patient died in a state suggesting pulmonary oedema shortly after admission Autopsy was not performed

Case 4 (P 17576) A 39 year-old woman admitted in March 1967 with a 3½ cm tumour in the right breast The tumour adhered to the skin No enlargement of regional lymph nodes Right sided mastectomy was performed and microscopic examination showed carcinoma The patient received postoperative irradiation and was castrated by radiation In March 1969 she was re admitted with a tumour of the left breast This tumour was 2 cm large, poorly defined but non adherent to the surroundings Simple mastectomy was carried out Microscopic examination showed carcinoma During this stay in hospital it was learnt that after the castration there had been menostasis until January 1969 Thereafter she had menstruation like bleedings of 4–5 days duration and in between spotting and post coital bleeding Through several years also daily yellowish brown vaginal discharge which had not increased recently Gynaecological examination showed the vaginal mucosa to be atrophic and readily bleeding There was tightening in both parametria and the uterus was small No other abnormalities were detected Chest radiography did not show any signs of metastases Microscopic examination of curettings showed carcinomatous metastases to the endometrium Treated with Prednisolone and X rays In July 1969 re admitted with acute retention of urine Digital examination of the rectum and vagina revealed that the true pelvis was moulded with neoplastic masses The patient died shortly after and no autopsy was done

Case 5 (186389) A 40 year-old woman admitted in February 1963 with uncharacteristic epi

gastric pain Laparotomy revealed a gastric tumour the size of a tangerine, about the middle of the greater curvature, and a Billroth II resection was done Histological examination disclosed carcinoma Postoperative course uneventful In May 1965 the patient was re admitted as a case of menopausal metrorrhagia Until the autumn of 1964 she had had regular periods, 7 days/28 days Thereafter, the periods grew somewhat irregular, and since February 1965 she had had long lasting bleedings of 4 weeks duration At admission she had a flat, fixed, hard mass, as large as an orange, in the epigastrium. The patient was anaemic, Hb level 65 On gynaecological examination granular infiltration was palpable in the anterior fornix, but not visible on inspection Fractionated curettage was carried out The cavity measured 8½ cm, was highly fibromatous and irregular Microscopic examination of a moderately large amount of normal looking curettings revealed carcinomatous metastases to the endometrial and cervical mucosa After the operation palliative irradiation was started The patient showed good tolerance of the irradiation but after discharge in July 1965 she went steadily downhill and died in August 1965 No autopsy

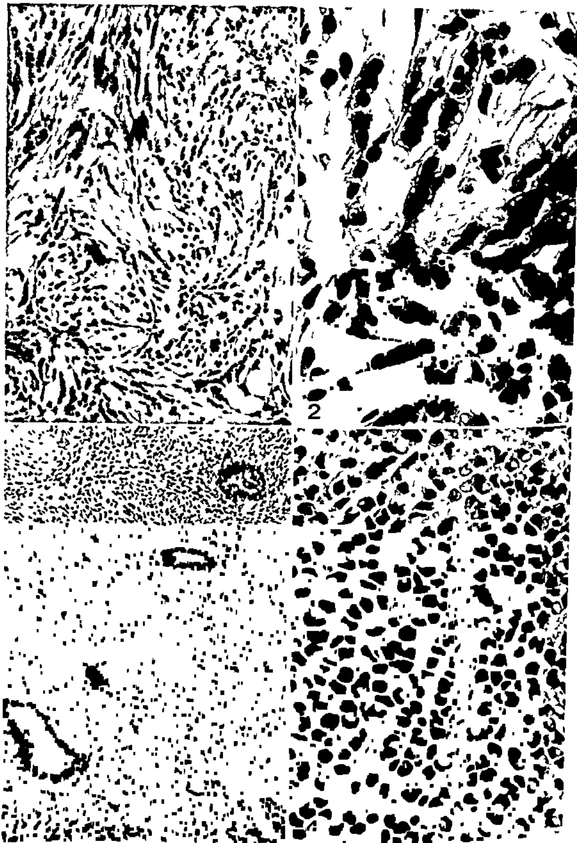
DISCUSSION

The primary tumours In one case the primary tumour was situated in the stomach It was a cellular, solid, colloid cancer with numerous signet ring cells, but without any extracellular accumulations of mucus The other 4 primaries were in all likelihood breast cancers All the patients had undergone operation for histologically confirmed breast cancer, and none exhibited symptoms or signs of primary tumours elsewhere Moreover, there were the special histological findings to be described below According to the literature (10, 15) tumours of the breast or stomach are the ones most likely to metastasize to the uterus Breast cancer is reported to make up 50 per cent of th primary tumours gastric cancer 20 per cen Other primary sources are rare (lung pleura pancreas liver, gall bladder, and malignant melanoma (6, 15))

Among our 4 breast cancers we succeeded in procuring the histological specimens for review from 3 (Cases 1 2, and 4) They were mutually of a striking similarity, being solid, scirrhous carcinomas differing from the commonplace types in the tendency of the epithelial cells to be arranged isolated or in short columns with one row of cells in each column (single filing) (Fig 1) The elements were moderately large, roundish, with a pale cytoplasm, vacuolized in places, and round nuclei with very few mitoses A few signet ring cells were found, and mucus stainings by alcian blue and mucicarmine were positive, but only in a small number of the cells Extracellular accumulation of mucus did not occur (Fig 2) The appearances were highly reminiscent of the special type of breast cancer which has a particular tendency to metastasize to smooth muscles (*epithelioma a cellules independantes et secretantes* or diffuse, scirrhous carcinoma) (1, 4), but differed in the scanty occurrence of signet-ring cells and in the faintly positive mucus staining Both in Vesterdal Jorgensen's and in Fischermann's cases autopsy disclosed metastases to the myometrium The endometrium is not mentioned The histological architecture might also suggest invasive lobular carcinoma, but this type of tumour does not secrete mucus and is as a rule combined with lobular carcinoma *in situ*

The endometrial metastases The curettings were not in any way striking on gross inspection They were small to moderately

tration was diffuse in 4 cases, but focal in one (Case 3) In the 4 diffuse cases the endometrial stromal cells were completely replaced by a very cellular tumour tissue, with no connective tissue, but with solid irregularly arranged epithelial cells which in the low power fields might be mistaken for normal stroma and with somewhat higher magnification for stromal sarcoma, but mucus stainings were positive, and there were numerous signet ring cells No extracellular mucus The glands of the endometrial mucosa were preserved Cytologically the tumour cells were of the same type as in the primary tumours, but whereas the breast cancers were hypocellular and showed only a few


2

signet ring cells, the reverse applied to the endometrial metastases (Figs 3 and 4) In the case where the tumour cells were of a more focal situation in the endometrium, they were somewhat reminiscent of the benign foam cells encountered occasionally in hyperplastic endometria (7) and in endometrial carcinoma (3), but stainings with mucicarmine and with alcian blue were positive The occurrence of signet ring cells in endometrial metastases has previously been briefly mentioned by *Stemmermann* (12)

Appearance of the myometrium In Case 1 subtotal hysterectomy was performed after the curettage The myometrium showed mild diffuse thickening, without gross focal changes Microscopy revealed diffuse neoplastic infiltration without well defined nodules The cells were of the same type as in the patient s breast cancer, with predominance of elements showing large, pale, central nuclei The tumour tissue was also more hypocellular than in the endometrial metastases, showing some isolated cells like the mammary tumour Mucus staining was faintly positive, and there was no extracellular mucus In Case 4 there were flakes of myometrium in the curettings, and the appearances were exactly like those in Case 1 In another two cases (Nos 3 and 5) there were small myometrial flakes without neoplastic infiltration

Clinical findings The first sign of metastases after operation for the primary tumour was abnormal vaginal bleeding in 4 cases In one of these patients however osseous metastases had been demonstrated by radiography shortly before The fifth patient (Case 2) had not at any time had vaginal bleeding but was admitted because of ascites

The interval from operation for the primary tumour until vaginal bleeding occurred ranged from less than 2 years to 5 years

The gynaecological examination in connection with the curettage disclosed no abnormality in one patient (Case 1), whereas the other 4 were found to show more or less definite signs of tumour in the internal genitals or true pelvis At this juncture 3 out of the 4 patients with previous breast cancer had no signs of metastases elsewhere In two osseous metastases were demonstrated 6 months and 2 years later

After the endometrial metastases had been diagnosed, 3 patients died in 6 months, 6 months, and 2 years Two are still alive, at 3 months and 18 months

CONCLUSION

Abnormal vaginal bleeding may be the first signs of metastases from an extrapelvic cancer, and the diagnosis may be based on curettings In cases where the primary tumour has been a breast cancer histological examination may show a special type reminiscent of *epithelioma à cellules independantes et secretantes* In 4 of the present cases the endometrial metastases showed great mutual similarity There were numerous signet ring cells, diffuse replacement of the stromal cells, and preserved endometrial glands In the fifth case the changes were focal, but otherwise of the same type Although the material is small, it seems justified to contemplate whether endometrial metastases occur particularly in cases of solid colloid carcinomas without extracellular accumulations of mucus

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Fig 1 Breast cancer (H & E × 125) (Case 1)

Fig 2 Breast cancer (H & E × 500) (Case 1

Fig 3 Metastases to the endometrium (H & E × 125) (Case 1)

Fig 4 Metastases to the endometrium (H & E × 500) (Case 1)

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Acta path microbiol scand Section A 80, 841-846, 1972

POST MORTEM CHANGES OF HUMAN SERUM ESTERASES

An Electrophoretic Study

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An attempt was made to follow post mortem changes of human serum esterases Disappearance of fractions and formation of new ones was demonstrated, using starch gel electrophoresis It is concluded that proteolytic enzymes may be the cause of these changes

A recent review (*Giblett* 1969) of esterases in human serum cites 95 publications, most of which deal with population studies and genetics Ontogenetic changes have been reported both quantitatively (*McCance et al* 1949, *Lehmann et al* 1957, *Harris et al* 1960) and qualitatively (*Harris et al* 1962) The present authors wanted to investigate how the serum esterases changed post mortem and whether these changes could be limited to a definite time scale *L dell et al* (1963) studied pseudocholinesterase activity in post mortem human tissues but this was done quantitatively so it did not reveal post mortem molecular types

The recent work of *Saeed et al* (1971) demonstrates how all iso enzymes of cholinesterase can be produced by the action of certain proteolytic enzymes on the major cholinesterase component cholinesterase (most likely C_4 of this work) under physiological conditions The above mentioned work encouraged us to carry out this study of post mortem changes of human serum esterases, and investigate whether these could be brought about experimentally

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MATERIALS AND METHODS

A horizontal starch gel electrophoresis was performed at 4 °C to separate the esterase fractions The gel buffer used was a triscitrate buffer at pH 7 6

(0 0143 M Tris (hydroxymethyl) methylamine 0 00406 M citric acid)

The vessel buffer used was a borate buffer at pH 8 6 (0 301M boric acid adjusted to pH 8 6 by adding NaOH) (*Arnason A* 1971)

Voltage applied was 17v/cm for 3 hrs

The gel slices were stained in 0 07M phosphate buffer at pH 6 0 The substrate used for most of the work was 1 napthyl acetate the coupling dye was Fast Garnet G B C (Gurr)

The staining was carried out at room temperature for up to 20 hrs and the gels were photographed at intervals as staining proceeded

Proteolytic enzymes were added to the serum and wholeblood and incubated at room temperature

Papain 5 mg/1 ml serum
Trypsin 1 mg/1 ml serum
Pepsin 5 mg/1 ml serum

Blood samples were collected with a syringe from the veins of the cubital fossa in live sub

TABLE 1 *Post Mortem Changes of Human Plasma or Serum Esterases*
(1 napthyl acetate used as substrate)

Fractions		Intra Vitam Normal plasma	Post mortem 2 hrs	5 hrs	10 hrs	20 hrs	40 hrs	80 hrs	100 hrs	720 hrs
IMFt	1		—	—			+		+	+
	2		—	—		±	+		+	+
	3		—	—		±	+	+	+ +	+ +
	4	±	+	+	+	+ +	+ +	+ +	+ +	+ +
A		+	+	+	+	±	±	±	±	±
C_1T			+	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
C_1		+ + +	+ +	+	±	(±)	(±)	(±)	—	—
C_2		+ +	+ +	+ +	+	±	(±)	—	—	—
C_3		+ +	+ +	+ +	+	+	±	(±)	—	—
5		+	+	+	+	+	+	+	+	+
C_4		+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
PMEII		—	—	±	+	+ +	+ +	+ + +	+ + +	+ + +

± faint + clear, + + strong + + + + very strong (±) sometimes present, — absent

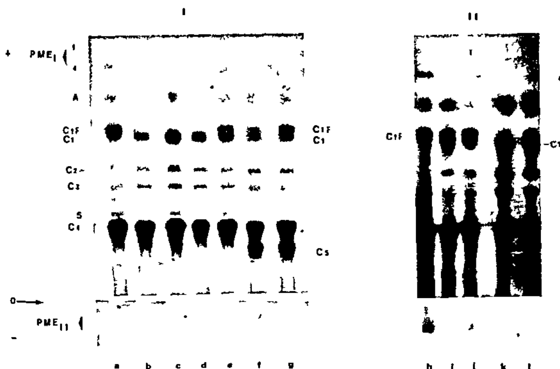


Fig 1, I and II Human plasma and serum esterase zymograms demonstrating post mortem changes of the esterase pattern

Gelbuffer Tris-citrate pH 7 6
Substrate 1 naphthyl acetate

a–e,	serum from 79 year old woman	a sample taken	11½ hrs post mortem
f g	serum from 62 year old man	b sample taken	½ hrs post mortem
h	serum from 51 year old man the body had been lying for 12 hrs at room temp before transfer to the cold room	c sample taken	1 hrs post mortem
		d sample taken	2 hrs post mortem
		e sample taken	4 hrs post mortem
i	serum from 72 year-old woman	f sample taken	2½ hrs post mortem
j	serum from 57 year old woman	g sample taken	5 hrs post mortem
k	control plasma from 22 year old man kept frozen for a month	h sample taken	24 hrs post mortem
		i sample taken	41 hrs post mortem
l	control plasma from a 40 year old woman kept at 4° C for 2 days	j sample taken	9 hrs post mortem
a j	post mortem sera or plasmas		
k l	plasma from live persons		

Nb Corpses were transferred to the cold room at 4° C six hours after death It will be stated where these conditions are different

jects but in the dead ones the blood was collected from the larger veins and in some cases from the heart These blood samples were then put into heparinized tubes and centrifuged The supernatant was transferred to another tube and deep-frozen The red cells were washed three times in an abundant amount of isotonic saline and then deep frozen All samples were stored at −20° C Sample holders were bits of Wattman filterpaper No 3 measuring 5 × 5 mm

RESULTS

Plasma from Living Subjects

The normal plasma and serum exhibited 6 esterase fractions when the above methods were employed, and the additional 7th fraction was found in some (c_5) Fractions C_1–C have been reported by several authors and

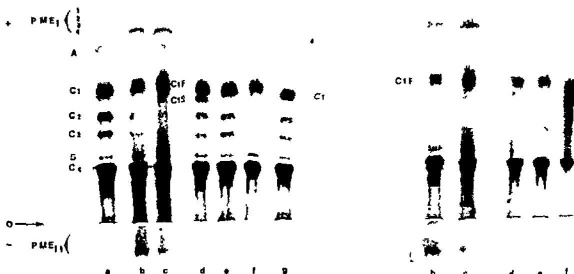


Fig 2 A zymogram showing effects of some proteolytic enzymes

Gelbuffer Tris citrate pH 7 6
Substrate 1 naphthyl acetate

Plate I
a, a fresh control plasma
b a post mortem serum 41 hrs afte death
c a post mortem serum 61 hrs after death
d,–j serum from the same individual
d after 3 hrs incubation with papain
e, after 3 hrs incubation with pepsin
f after 3 hrs incubation with trypsin
g *after 3 hrs incubation with water (equal volume)*

Plate II
h the same as d, but incubated for 27 hrs
i the same as e, but incubated for 27 hrs
j the same as g but incubated for 27 hrs

fraction 5 is probably fraction ChE_4 of *Saeed et al* (1971)

Fraction 4 in the PME_I zone was found in all plasmas tested but was always weak and needed at least 4 hrs to develop This fraction can not be observed if a Tris citrate gel at pH 8 6 is used Storage of the plasma at room temperature for 15 days did not alter the zymogram greatly nor did the plasma change when stored frozen for longer periods except for "storage bands slower than C_1 described earlier (*Harris et al* 1962)

Post Mortem Sera and Plasmas

120 post mortem blood samples were examined Sampling time after death ranging from ¼ hour to 720 hours In some cases samples were taken from the same body at intervals Various changes of the esterases seem to occur after death and some of them are described here

For these changes see Table 1 and Figs 1 2 and 3

Formation of Zone PME_I The first change occurs when fraction 4 of this zone becomes stronger and continues to increase in strength for at least 100 hours following death, the strength remains at the same level a month after death (1 sample) *Fraction 1* in this zone is easily visible (to the eye) 11 hrs after death, fractions 2 and 3 though visible are faint 20–30 hrs after death

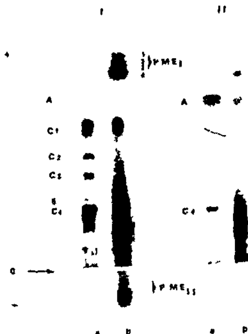


Fig 3 Two zymograms to compare two substrates 1-naphthyl acetate (I) and Napthol AS acetate (II)

a a plasma sample, from a 31 year old man preceding death stored at room temp for 5 days

b post mortem serum from the same man as a, taken 95 hours following death The body had been kept at ca 10° C for 3 days

Formation of Zone PMEII There are individual differences in the intensity of this zone, but in all cases tested, the zone could be demonstrated between 8 and 9 hours after death

Changes of Esterase Band C_1 C_1 seems to start forming a new and faster moving fraction C_1F which may be detected as early as 2 hours after death Fig 1) Four hours following death C and C F have a similar intensity but 5 hours af [illegible] death C_1F is usually stronger than C_1 In some cases C_1F has completely substituted C after 12 hours but in other cases rema[illegible] of C_1 may be detected up to 90 hours after death

Disappearance of Fra[illegible]ons C_4 and C_3 Following death thes[illegible] fractions disappear gradually C usually [illegible] aring first The earliest complete disa[illegible]nce found was 21 hours after death and the latest disappearance recorded was 90 hours after death

In highly haemolysed samples, when the body had been kept at room temperature for a long period, a strong esterase smear was frequently found, spreading from the position of C_4 to the line of application In these samples zone PMEI and PMEII were also prominent

When red cell lysates were run on the same plates, red cell esterases did not overlap any of the serum fractions described earlier

Changes when Napthol-AS-acetate was used as substrate The albumin zone and C_4 reacted to Napthol-AS-acetate (*Augustinsson* 1958, *Harris et al* 1962) in the control plasma samples The post mortem samples show different behavior, as fraction 4 of zone PMEI breaks down this substrate, but the reactivity of the albumin (A) towards this substrate seems to be decreased (Fig 3) No changes can be observed in the nigrosine-amidoblack stained half of the gel A diffuse zone reaching from the line of application to C_4 is also stained when Napthol-AS-acetate was used

The post mortem changes of aryl esterases are therefore a formation of a new aryl esterase fraction at PMEI 4, at the same time fraction A decreases

Experimental Changes of the Plasma The effects of papain pepsin and trypsin are demonstrated in Fig 2 As expected the proteolytic enzymes attack the esterases and change the mobility of the fractions (*Saeed et al* 1971) The effect of trypsin after 3 hrs of incubation at room temperature is similar to what happens in death—C_4 and C_3 disappear and C_1 takes up the position of C_1F

The *papain* effect was similar but slower and it formed a new fraction C S *pepsin* acts similarly to trypsin, but more slowly and does not form C_1S Incubation of sera at 50° C for 15 minutes destroyed all esterases But storing the sera at room temperature for up to 3 weeks did not alter the pattern except for formation of new fractions which

migrated behind C_4—apparently the storage bands reported by *Harris et al* (1962)

DISCUSSION

The results of the present investigation show that the plasma or serum esterase pattern changes after death The storage of blood outside the body does not change in the same manner, as if it is kept "inside' the body after death The question arises which factors could there be present in the blood post mortem causing these alterations of mobility and quantity of the esterase fractions? As mentioned previously in this article, *Saeed et al* (1971) demonstrated the break down of certain cholinesterase component by proteolytic enzymes *in vitro,* thus forming the other iso enzymes of the plasma Similar experimental changes are reported here using certain proteolytic enzymes The suggestion is therefore made that fractions C_1, C and C_5 at least are affected by proteolytic enzymes which are released after death, or alternatively inhibited while the person is alive As the esterase fractions were not labelled premortem one cannot tell their fate with any certainty nor the origin of the "post mortem esterases" (PME) We could suggest, however that the aryl esterase at the albumin site (A) is broken down post mortem and takes up a new position at PMEI, 4

The PMEI zone (post mortem esterase zone I) is possibly a proteolytic break down product of other esterase isozymes in the blood although this zone could not be produced experimentally

The PMEII zone (post mortem esterase zone II) can hardly be a break down product of the plasma esterases This conclusion is based on the fact that a serum obtained at intervals from the corpse of a woman who had a widespread metastatic carcinoma of the liver showed only very faint esterase fractions, but had the usual appearance of PMEII zone (*McArdle* 1940)

The conclusion of present investigation is that there is a proteolytic break down of the plasma esterases following death, and that new molecular types are formed, some of these new types are break down products of the plasma esterases, but others are of a different origin

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We want to express our gratitude to the staff of the *University Hospital Reykjavik* and the *Department of Pathology, University of Iceland Reykjavik,* for their help

This work has been supported by a grant from *Atomic Energy Commission,* Contract No AT (30 1) 3548

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QUANTITATIVE DETERMINATION OF BLOOD GROUP SUBSTANCES A OF ORAL EPITHELIAL CELLS BY IMMUNOFLUORESCENCE AND IMMUNOPEROXIDASE METHODS

E Dabelsteen

Variation in the expression of blood group antigen A of oral squamous epithelial cells was demonstrated by serial two fold titrations with immunoperoxidase (IP) and immunofluorescence (IF) staining methods Cells from 6 non secretor individuals and 15 secretor individuals all belonging to blood group A_1, were tested Cells from non secretor individuals reacted weakly with IF staining and negatively with IP staining Cells from secretors reacted with both staining methods and with much higher endpoint titres than the non secretor cells A considerable variation in endpoint titres was found in both groups

The presence of blood group substances A and B in cells and tissues other than the erythrocytes is well documented (17, 18, 30) An excellent review of the early literature is given by *Hartmann* (13)

In the oral mucosa their localization on the cell membranes of the epithelial cells was demonstrated by *Brandtzaeg* (2), *Holborow et al* (14), and *Szulman* (25)

A partial or complete loss of the blood group substances has been reported to occur in neoplasms originating from epithelium in which substances are normally present (5 7, 8, 9, 10, 15, 16, 20), and it has been suggested (5, 8) that the loss of blood group antigen may be used in the early diagnosis of malignant changes In order to investigate this possibility further studies describing the histological distribution and possible quantitative variation in normal tissue are needed, so that a standard against which a loss can be evaluated, can be established This is of importance since the strength of A and B blood group substances of normal human erythrocytes from different individuals is known to vary (3, 4, 11, 12, 19) and it appears that a similar variation may be expected on cell surfaces of oral (24) and cervical epithelia (9) and tissue from the pancreas (10)

The purpose of the present study was to investigate a possible quantitative variation of blood group antigen A in normal buccal tissue so that a base line level, against which malignant changes can be evaluated, can be established

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MATERIAL AND METHODS

The material consisted of biopsies from normal oral buccal mucosa of 21 young healthy individuals all

belonging to blood group A_1. Fifteen were classified by identification of the blood group A substances in the saliva as secretors and 6 as non-secretors. The secretor status was estimated by a haemagglutination inhibition test following the principles described by *Hartman* (13). All the non-secretors had a titre of 0. Among the secretors the lowest titre was found to be 64 and the highest 2048. Blood grouping was performed at the Blood Grouping Department, Statens Seruminstitut, Copenhagen and the secretor status at the Forensic Institute, Department of Serology, Copenhagen. The biopsies were fixed in 10 per cent neutral formalin for 24 hours at 4° C, paraffin embedded and serially sectioned at 5 microns.

Blood group substances were detected by two different immunologic staining procedures. The immunoperoxidase (IP) method (1) and the immunofluorescence (IF) method (27). Both staining methods were used as double layer techniques, the first layer being a human anti A serum and the second layer a rabbit antihuman IgG globulin conjugated with peroxidase for the IP staining and with fluorescein isothiocyanate (FITC) for the IF staining.

The anti A serum was an anti A test serum purchased from Hoechst, Frankfurt, Germany. Both conjugates were provided by the Protein Laboratory, University of Copenhagen.

The peroxidase conjugate had a molar peroxidase/protein ratio of 1.20 and an antibody titre of 320 (*i.e.* 1 ml conjugate absorbs 320 μg pure human IgG). A working titre for this conjugate of 1:40 was obtained by chess board titration (28). The FITC conjugate had a protein concentration of 5 mg/ml (29), the anti IgG titre being 200. The optical density ratio at 495 nm/280 ranged from 0.95 to 0.30 with a mean of 0.66 (unconjugated immunoglobulin molecules, optical density below 0.30 and conjugated immunoglobulins with optical density ratio above 0.95 were removed by ion exchange chromatography) (26). By chess board titration (28) a working titre of 1:40 was found.

A Leitz Ortoplan® fluorescence microscope modified with a Tiyoda® wide angle darkfield oil immersion condenser was used. The light source was an Osram HBO lamp. Sections were studied at a magnification of 200×, using a 25× plane fluorite objective NA 0.5 and periplan eyepiece 8×. The primary filter was a FITC interference filter with red contrast band (21, 22), (Laboratory for Technical Optics, Lyngby, Denmark). The secondary filter was a 2 mm glass filter (Schott & Gen., Mainz, Germany) matched to fit the primary filter (21, 22).

The relative amount of blood group substance in tissue sections was estimated by a two fold serial dilution titration of the anti A. Reactions were registered as positive or negative and the *reciprocal of the highest dilution yielding positive* reaction was regarded as the endpoint titre.

To check on the reproducibility of the staining reaction all titration series were performed in triplicate. A second biopsy was taken from nine of the individuals for the purpose of testing the intra individual variation in the endpoint estimation.

To be sure that only cells at the same stage of differentiation were compared all readings were done in the upper part of the spinous layer. Control reactions served to ensure that the staining reactions were specific. These are summarized in Table 1.

RESULTS

In secretors, the cell membranes of the entire spinous layer stained uniformly positive. The planocellular layer reacted except for the surface 1–2 rows of cells completely nega-

TABLE 1. *Controls for Establishing Specificity of Immunoperoxidase and Immunofluorescen Staining*

Blood group substance	Blood group antisera	Conjugate	Results
A	Phosphate buffered saline	Labelled antiglobulin	Negative
A	Anti B	Labelled antiglobulin	Negative
A	Anti A absorbed with A_1 erythrocytes	Labelled antiglobulin	Negative
B	Anti A with known reactivity	Labelled antiglobulin	Negative
A*	Anti A with known reactivity	Unlabelled antiglobulin followed by peroxidase reaction	Negative
A	Anti A with known reactivity	Labelled antiglobulin	Positive

* For immunoperoxidase stainings only.

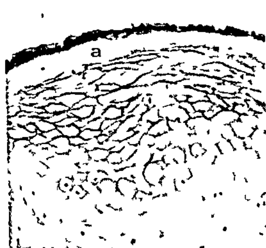


Fig 1 Normal buccal mucosa from a secretor individual Immunoperoxidase staining Note the negative reacting zone (a) between the heavily stained outermost cell layers and the spinous cells where the black intercellular spaces indicate the presence of blood group antigen A × 160

tively or very weakly The basal 2–3 layers were always negative (Fig 1) The pattern was the same in the non secretors except that the planocellular layer always reacted negatively even on the surface

It is seen (Fig 2) that it was possible by the IF staining technique to demonstrate blood group antigen in the epithelium in all 21 individuals tested with the IP staining all the non secretors reacted negatively The secretors were all positive but with lower titres than in the IF staining (2–3 two fold titre steps), (Fig 3) It is furthermore seen that the amount of antigen in secretors is high while in the non secretors it is low and sometimes at the limit of what can be measured Table 2

A Mann Whitney U test (23) seems to indicate that the secretors and the non secretors in respect to amount of blood group antigen in the epithelium are two different groups ($P < 0\,001$) From the triple titrations (Table 2) it is noted that the intra individual variation observed in the triple IF stainings ($S - 0\,25$ d f $- 42$) is significantly less than the interindividual variation in both groups ($P < 0\,001$) (non secretors having $S = 5\,0$ d f 5 and secretors $S^* = 1\,9$ d f 14) Furthermore it is seen from Table 3 which demonstrates endpoint titres obtained in repeated biopsies from the same location of the same individual that it has not been possible to show intra individual variations (S $0\,11$ d f 9) other than those which can be explained by variation in the staining technique This signifies that the variation in endpoint titres within the groups represents biological variation and not a chance variation such as might be expected

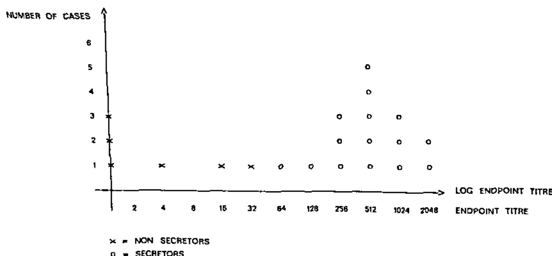


Fig 2 Endpoint titres obtained in the immunofluorescence staining of oral epithelial cells

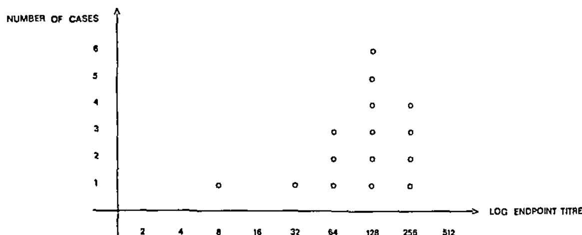


Fig 3 Endpoint titres obtained in the immunoperoxidase staining of oral epithelial cells from secretor individuals

among several random samples from the same population

DISCUSSION

The present work confirms and quantitates the variation among different individuals in reactivity of blood group substances A on normal oral squamous epithelial cells Furthermore, the study has shown that a study of the distribution of blood group antigen in tissue sections requires a carefully selection of staining techniques The sensitivity must be high, if the less sensitive technique is

TABLE 2 *Endpoint Titre Obtained by the IF and IP Techniques*

Case no	Endpoint titre IF 1	IF 2	IF 3	IP 1	IP 2	IP 3	Secretor *status*
47 518	1 4	1 4	1 4	0	0	0	—
47 918	1 16	1 16	1 16	0	0	0	
48 061	None	None	0	0	0	0	—
48 576	1 16	1 32	1 32	0	0	0	
48 043	None	1 2	None	0	0	0	
48 120	None	None	None	0	0	0	—
47 475	1 512	1 512	1 1024	1 128	1 128	1 128	+
47 410	1 1024	1 2048	1 2048	1 512	1 256	1 256	+
47 970	1 32	1 64	1 64	1 8	1 8	1 8	+
47 760	1 512	1 256	1 256	1 128	1 64	1 64	+
47 759	1 128	1 256	1 128	1 32	1 64	1 32	+
47 761	1 512	1 512	1 1024	1 128	1 64	1 128	+
48 041	1 256	1 256	1 512	1 64	1 128	1 128	+
48 064	1 256	1 256	1 512	1 64	1 64	1 128	+
48 063	1 512	1 512	1 512	1 64	1 64	1 64	+
48 062	1 512	1 512	1 1024	1 256	1 64	1 64	+
48 090	1 1024	1 2048	1 2048	1 256	1 512	1 256	+
48 092	1 512	1 512	1 256	1 128	1 128	1 128	+
48 105	1 1024	1 1024	1 2048	1 256	1 128	1 256	+
48 577	1 512	1 1024	1 1024	1 128	1 64	1 128	+
48 044	1 51	1 1024	1 512	1 256			+

TABLE 3 *Endpoint Titres Obtained by Titration in the IF Staining Technique of Tissue from the Cheek Obtained at an Interval of about Two Months*

Pat ent	Blood type	Cheek 1	Cheek 2
47410	A_1	1 2048	1 2048
47970	A_1	1 64	1 32
47760	A_1	1 256	1 256
47759	A_1	1 128	1 128
48064	A_1	1 512	1 128
48105	A_1	1 1024	1 1024
48041	A_1	1 256	1 256
4806?	A_1	1 512	1 512
49584	A_1	1 128	1 128

used (the IP technique) non secretor cases would show negative reaction in malignant as well as normal epithelium, and would be of little value as a diagnostic aid Further more the reproducibility must be high since comparison between normal and malignant tissue would otherwise be extremely difficult especially for the non secretors which in many cases again would react negatively in malignant as well as normal tissue

The reason for selecting formalin fixation and paraffin embedding for the investigation of the blood group substances in oral epi thelium has been discussed in a previous paper (6), but it should be mentioned here that it has been stated (16) that blood group substances due to their polysaccharide na ture, are not influenced by formalin fixation and paraffin embedding By demonstrating the presence of antigen bound to epithelial cells in the non secretor group the present work has confirmed previous studies (6 24 25) which have shown that the origin of the blood group antigen on the epithelial cells is independent of that found in saliva This is further supported by the finding that the planocellular layer reacted negatively or ex tremely weak in the secretors as well as the non secretors The negative planocellular layer seems to exclude the possibility that the blood group antigen present in the spinous layer is absorbed from the saliva This to gether with the negative reacting basal layer suggests that all the blood group antigen present in the spinous layer is produced in the epithelium

The study has shown that the secretors and the non secretors have not only a different amount of blood group antigen in saliva but in the buccal mucosa as well Furthermore the study has shown that a biological varia tion in amount of blood group antigen are likely to exist within both groups These findings support results previously published by *Swineburne et al* (24) By using the mixed cell agglutination technique which does not lend itself to quantitation these authors (24) were able to show variation in the expression of blood group antigen A on buccal cells between a group of non secretors and a group of secretors, and furthermore a slight variation within the secretor group But as they used exfoliated cells the variation was in part felt to be due to an adsorption of antigen from the saliva of the secretors onto the cell surfaces (24) and thus could have accounted for some of the differences found

The present study has shown that it is not possible to use a general standard if the loss or decrease in amount of blood group sub stances is to be used in the early diagnosis of malignant changes as it has previously been suggested (5 8) The amount of blood group substances in the patient s normal mucosa must in every case serve as base line against which malignant changes are evaluated

The relationship between the amount of blood group antigen in saliva and the amount of antigen in the spinous cell layer in the oral mucosa as not investigated This relation ship should be studied as it may be of future use in tests where an individual base line has to be established However it would not be of interest until the next step in the work concerning the problems of establishing a base line which is to investigate whether there is a variance in reactivity in different locations in the oral cavity has been finished

If a site to site variation of amount of antigen is found a saliva sample would probably be of little value

In conclusion the present study showed that the reactivity of blood group antigen of oral squamous epithelial cells varies among different individuals This is an important factor to consider when the degree of reactivity in malignant and premalignant tissue has to be investigated

The present work was supported by *Daell Fonden', Copenhagen, Denmark* and Grant DE 1358 from *The National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U S A*

The author wants to thank *Dr J Andersen* head of Blood Grouping Department, Statens Seruminstitut, Copenhagen and *Dr K Henningsen*, Department of Serology University Institute of Forensic Medicine, Copenhagen for kindly assistance, *Chemist Agnete Ingild*, Protein Laboratory, University of Copenhagen for kindly providing conjugates and *cand act M Nyholm* for advice in the statistical part of the study

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ON THE INFLUENCE OF NEURAMINIDASE ON CASEIN-INDUCED AMYLOIDOSIS IN C_3H MICE

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Intraperitoneal inoculation of the receptor-destroying enzyme neuraminidase had little effect on spleen amyloid development in mice during caseination However, incubation of normal syngeneic lymph node cells with neuraminidase before they were injected into mice during casein treatment significantly enhanced the amyloid promoting effect of the cells It is suggested that neuraminidase treated lymph node cells may be preferentially trapped in the spleen with subsequent transfer of an amyloid component to macrophages The polycation DEAE dextran known to accelerate amyloid formation if given intraperitoneally with casein, did not influence the effect of cell transfer This may indicate that the amyloid promoting mechanisms of the two membrane active substances are different

Two polycations DEAE dextran and Polybren, if inoculated intraperitoneally (i p) or subcutaneously (s c), have been shown to enhance amyloid formation in caseinated mice Injections of dextran were less effective and the anion dextran sulphate was completely without effect (*Ebbesen* 1972) Although the mechanism whereby polycations exert an effect is not known it seems likely that it is related to cell membrane charge

Some activities of the enzyme neuraminidase on cells have been described recently in particular those concerning its ability to alter charge density (*Vasudenan et al* 1970) and to unmask certain antigenic determinants (*Currie & Bagshawe* 1968)

In anticipation of a possible similarity of action of polycations and neuraminidase during amyloid development neuraminidase alone, neuraminidase treated lymph node cells, and DEAE dextran treated cells have been injected into mice during caseination

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MATERIAL AND METHODS

Two month old inbred C_3H female *mice* fed on mouse pellets and water ad libitum were used

Casein was administered with 0 5 ml of a 5 per cent solution subcutaneously 5 times a week for 6 weeks Neuraminidase (Vibrio Cholerae Behring werke) was diluted in Minimum Essential Medium (Eagle) pH 7 2 (MEM) Half a ml containing 14 international units was injected intraperitoneally simultaneously with each casein inoculation

Donor cells were harvested from mesenteric lymph nodes of two month-old inbred C_3H female mice and washed twice in MEM

Half the cells were made up to $1\ 5 \times 10^7$ cells per ml in MEM containing 25 units neuraminidase ml and incubated for 30 min at 37° C The other half *of the cells* was incubated in MEM only

After washing in MEM 0 5 ml of suspension containing $1\ 5 \times 10$ cells was inoculated intravenously into each mouse on days 7, 14, 21, 28 and 35

The percentage of dead leucocytes was deter

TABLE 1 *Development of Spleen Amyloid in Caseinated C_3H Mice Modified by Neuraminidase Treatments*

Experimental procedure	No of mice	Spleen amyloidosis Mean	Range	P	Casein antibodies Radial diffusion diameter in mm
Casein s c	14	2 6	(2–3)		3 7
Neuraminidase i p	10	0			
Casein s.c + Neuraminidase i p	15	3 0	(2–4)	$0\ 001 < p < 0\ 01$	3 5
Lymph node cells i v	10	0			
Casein s c + lymph node cells incubated in MEM i v	13	3 0	(2–4)	$0\ 001 < p < 0\ 01$	4 0
Casein s c + lymph node cells incubated in MEM with neuraminidase i v	16	3 6	(2 5)	$p < 0\ 001$	3 8
Casein s c + lymph node cells incubated in MEM with DEAE dextran i v	10	2 6	(2 3)		3 4

mined for each cell suspension by the methylen blue dye exclusion test prior to and after the incubation

About 20 per cent of the inoculated cells were dead irrespective of the presence of absence of neuraminidase or DEAE-dextran in the incubating medium

All animals were killed on day 42 The lung, liver, spleen, kidney, mesenteric lymph node, peripheral lymph node, thymus and thyroid gland were taken for microscopy and stained with Periodic acid Schiff (PAS) and alkaline Congo red. Amyloid was identified by its birefringence with Congo red under crossed polars

The degree of spleen amyloidosis was determined on PAS stained sections (*Christensen & Hjort 1959*) A continuous ring of amyloid around spleen folicles being rated as grade 3

Antibodies to casein in the serum harvested at autopsy were determined by a modification (*Ebbesen* 1971) of the single radial diffusion technique Wells of 2 mm in diameter in an agar gel containing 1 per cent casein were filled three times with 2 µl of serum and then incubated for 3 days

RESULTS

Results from the various amyloidosis inducing schedules are given in Table 1

If a small amount of amyloid was present it would be confined to the spleen Grade 4 of spleen amyloid was accompanied by liver amyloid but no amyloid was observed in other organs

It can be seen that neither neuraminidase nor untreated lymph node cells induced amyloid if given to otherwise untreated mice (Table 1)

Mice that received either neuraminidase or untreated cells with casein had slightly more amyloid than mice treated with casein only

Transfer of lymph node cells pretreated with neuraminidase to mice undergoing casein treatment significantly enhanced the amyloid formation However, transfer of cells pre-incubated in DEAE dextran did not affect amyloid formation

Antibodies to casein apparently occurred with the highest concentration in mice grafted with untreated lymph node cells

DISCUSSION

Neuraminidase will remove N-acetylneuraminic acid (*Gottschalk* 1959) and thereby render cells insusceptible to certain viruses (*Rowe* 1961) Under certain conditions, however, neuraminidase may enhance infection (*Ebbesen* 1972)

Treatment with receptor destroying enzyme

is also known to increase the sensitivity of the cytotoxic test (*Vasudenan et al* 1970)

Since casein has an isoelectric pH below 7 4 (*Haurowitz* 1963) it should attach more readily to cell surfaces the charges of which have been elevated by neuraminidase treatment (*Vasudenan et al* 1970) This mechanism may be of some importance since intraperitoneal administration of neuraminidase had a marginal effect

However, pretreatment of lymph node cells with neuraminidase did significantly increase the formation of amyloid

Two explanations of this phenomenon can be given Firstly Neuraminidase treatment of cells may expose otherwise concealed antigenic sites (*Vasudenan et al* 1970) and thereby increase the antigenic load (*Currie & Bagshawe* 1968) of the casein treated animals, and in some way enhance deposition of amyloid

Secondly We have previously observed amyloid fibrils in mouse lymph node cells (*Ebbesen et al* 1969) where amyloid is never detectable by light microscopy It is therefore possible that amyloid fibrils or their precursors may be produced in the lymph nodes and be carried by cells from there to spleen macrophages (*Ebbesen* 1971, *Hardt et al* 1972) Any interaction between the injected lymph node cells and macrophages may be enhanced by the increased positive charge of the former after neuraminidase treatment

As in previous experiments (*Ebbesen* 1971) there is no correlation between degree of amyloidosis and level of circulating antibodies to casein

The lack of effect of DEAE-dextran transferred lymph node cells indicates that polycations and neuraminidase influence different processes involved in amyloid formation

This investigation was supported by *Anders Hasselbalcks Fond til Leukæmiens Bekæmpelse*, and *Daell Fonden*

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